Enzymatic and genetic studies on microbial NAD kinase and its application(Dissertation_全文)

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CONTENTS

INTRODUCTION

NAD and NADP are compounds of great importance in cellular metabolism and are known to participate in more than 440 enzymatically catalyzed oxidation-reduction reactions (1). NAD is involved primarily in catabolic or degradative reactions, while NADP functions in anabolic or biosynthetic reactions requiring reducing power to drive the reaction (2). NADP is now regarded to be formed through the phosphorylation of NAD by the use of ATP (3) and this phosphorylation is catalyzed by NAD kinase (ATP:NAD phosphotransferase, EC 2. 7. 1. 23):

$$NAD + ATP \rightarrow NADP + ADP$$

The enzyme has only been completely purified from pigeon liver (4), pigeon heart (5), *Saccharomyces cerevisiae* (6), and *Candida utilis* (7), and some properties of the partially purified enzymes have been reported (3, 8). However, since no NAD kinase gene has been cloned yet, several unresolved questions remain, especially as to the structure of this enzyme (3).

On the other hand, although NAD kinase had been well accepted to utilize ATP or other nucleoside triphosphates as a sole source for phosphorus (3, 8), Murata *et al.* found inorganic polyphosphate [poly(P)]-dependent NAD kinase activities utilizing poly(P) for the phosphorylation of NAD (9) in some bacteria:

$$NAD + poly(P)n \rightarrow NADP + poly(P)n\text{-}1$$

Poly(P) is a polymer of inorganic orthophosphate residues (Fig. 1) linked by high-

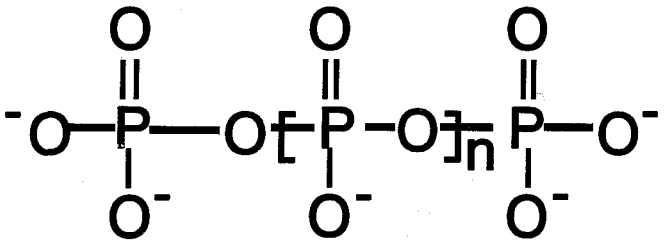

FIG. 1 Structure of poly(P) with the chain length of n + 2.

energy phosphoanhydride bonds, and is now regarded as present in nearly all classes of living things, from bacteria to mammals (10). Knowledge as to the poly(P)-dependent NAD kinase has been greatly limited, since even an isolation of the enzyme has not been achieved so far.

In contrast to poly(P)-dependent NAD kinase, some poly(P)-metabolizing enzymes and

their genes including poly(P) kinase, poly(P) phosphatase, and poly(P)/ATP-glucokinase have been isolated and understandings as to these enzymes have greatly improved in this decade (Table 1).

TABLE 1 Reactions catalyzed by poly(P)-metabolizing enzymes

Enzymes	Reactions
Poly(P) kinase	poly(P)n-1 + ATP $\rightleftarrows$ poly(P)n + ADP
Poly(P) phosphatase	poly(P)n $\rightarrow$ poly(P)n-1 + orthophosphate
Poly(P)/ATP-glucokinase	glucose + $\begin{pmatrix}\text{poly(P)n}\\ \text{ATP}\end{pmatrix}$ $\rightarrow$ glucose-6-phosphate + $\begin{pmatrix}\text{poly(P)n-1}\\ \text{ADP}\end{pmatrix}$

Poly(P) kinases were purified from *Escherichia coli* (11), *Neisseria meningitidis* (12), *Propionibacterium shermanii* (13), and *Acinetobacter* sp. (14) and their genes were cloned from *E. coli* (15), *N. meningitidis* (16), *Pseudomonas aeruginosa* (17), and *Klebsiella aerogenes* (18). Poly(P) phosphatases were isolated from *S. cerevisiae* (19 - 21) and the genes were cloned from *S. cerevisiae* (23), *E. coli* (24), and *P. aeruginosa* (17, 25). Poly(P)/ATP-glucokinases were purified from *P. shermanii* (26) and *Mycobacterium tuberculosis* (27), and the gene was cloned from *M. tuberculosis* (28).

In this thesis, an enzyme responsible for poly(P)-dependent NAD kinase activity was isolated for the first time from *Micrococcus flavus* and revealed to be a novel enzyme designated as "poly(P)/ATP-NAD kinase" that can utilize poly(P) as well as ATP (Chapter I Section 1):

$$\text{NAD} + \begin{pmatrix}\text{poly(P)n}\\ \text{ATP}\end{pmatrix} \rightarrow \text{NADP} + \begin{pmatrix}\text{poly(P)n-1}\\ \text{ADP}\end{pmatrix}$$

NAD kinase that utilizes ATP, but not poly(P):

$$\text{NAD} + \text{ATP} \rightarrow \text{NADP} + \text{ADP}$$

was also isolated from *E. coli* and named "ATP-NAD kinase" to distinguish it from poly(P)/ATP-NAD kinase (Chapter II Section 1). Furthermore, genes encoding poly(P)/ATP-NAD kinases were cloned from *M. flavus* (Chapter I Section 2) and *M. tuberculosis* H37Rv (Chapter III Section 1) and those of ATP-NAD kinases were isolated from *E. coli* (Chapter II Section 2) and *S. cerevisiae* (Chapter III Section 2). They were the first reports on cloning of microbial NAD kinase genes. Properties (Chapter III Section 2)

and molecular structures (Chapter III Section 3) of these NAD kinases are discussed. Successful application of poly(P)/ATP-NAD kinase to the industrial NADP production was demonstrated (Chapter IV).

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Chapter I

Studies of Inorganic Polyphosphate/ATP-NAD Kinase of *Micrococcus flavus*

Section 1 Purification of a novel enzyme, inorganic polyphosphate/ATP-NAD kinase from *Micrococcus flavus*

NAD kinase (ATP:NAD phosphotransferase, EC 2. 7. 1. 23) catalyzes the only (known) biochemical reaction leading to the formation of NADP from NAD by the use of ATP (1):

$$NAD + ATP \rightarrow NADP + ADP$$

Completely purified NAD kinase has been obtained, only from pigeon liver (2), pigeon heart (3), *Saccharomyces cerevisiae* (4), and *Candida utilis* (5), while the gene encoding the enzyme has not been cloned from any organisms.

Although it had been well accepted that NAD kinase utilizes ATP or other nucleoside triphosphates as a phosphoryl donor, Murata *et al.* found inorganic polyphosphate [poly(P)]-dependent NAD kinase activities in some bacteria, especially in genera of *Micrococcus* and *Brevibacterium* (6). Poly(P)-dependent NAD kinase catalyzes the phosphorylation of NAD by the use of poly(P) (6):

$$NAD + poly(P)n \rightarrow NADP + poly(P)n\text{-}1$$

Poly(P) is a polymer of inorganic orthophosphate residues linked by high-energy phosphoanhydride bonds, and is now regarded as present in nearly all classes of living things, from bacteria to mammals (7). Studies of poly(P) have greatly improved in this decade through isolating poly(P)-metabolizing enzymes and its genes including poly(P) kinase, poly(P) phosphatase, and poly(P)/ATP-glucokinase (8-25).

However, an enzyme responsible for poly(P)-dependent NAD kinase activity, as well as a gene coding for the enzyme, has never been isolated hitherto. Even a simple question has been unsolved whether a single enzyme catalyzes only a poly(P)-dependent NAD phosphorylation or both poly(P)- and ATP-dependent NAD phosphorylations like poly(P)/ATP-glucokinase that catalyzes both poly(P)- and ATP-dependent glucose phosphorylations (23, 24). To elucidate the properties of the enzyme responsible for poly(P)-dependent NAD phosphorylation and to obtain the gene coding for the enzyme, NAD

kinase was purified from *Micrococcus flavus*, found to catalyze both poly(P)- and ATP-dependent NAD phosphorylations:

$$\text{NAD} + \begin{pmatrix} \text{poly(P)n} \\ \text{ATP} \end{pmatrix} \rightarrow \text{NADP} + \begin{pmatrix} \text{poly(P)n-1} \\ \text{ADP} \end{pmatrix}$$

and was designated as "poly(P)/ATP-NAD kinase". This is the first report on identification and characterization of poly(P)/ATP-NAD kinase.

MATERIALS AND METHODS

Strains. A cultivation condition of *M. flavus* (IFO 3242) was described in the text.

Assays. Poly(P)- and ATP-dependent NAD kinase activities were assayed as described (6) in a reaction mixture (1.0 ml) consisting of 5.0 mM NAD, 5.0 mM MgCl2, 100 mM Tris-HCl (pH 7.0), phosphoryl donor [poly(P) and 5.0 mM ATP, respectively] at 37 ℃, and NADP formed was enzymatically determined with isocitrate dehydrogenase (6). When poly(P)- and ATP-dependent NADH kinase activities were assayed, NAD was replaced by 5.0 mM NADH in a reaction mixture (0.10 ml). Reaction was terminated by adding 10 μl of 200 mM EDTA, and to the mixture 10 μl of 1.0 M glycine-NaOH was added to stabilize reduced nucleotides. NADPH formed was determined by thin layer chromatography (TLC) and HPLC as follows. Sample (5.0 μl) was spotted on a silica gel, and developed with a solvent [isobutyrate – 500 mM NH4OH (5:3 v/v)] which definitely separates nucleotide compounds (6). As controls, authentic NAD, NADH, NADP, NADPH, and ATP were also spotted. The developed nucleotides were detected with exposure to UV light at 312 nm. When HPLC was performed, sample (5.0 μl) filtered with Millex filter (0.45 μm; Millipore, Bedford, MA) was applied to a Cosmosil packed column 5C18-AR-II (0.46 x 15.0 cm) (Nacalai Tesque, Kyoto, Japan) and the adsorbed nucleotides were eluted through an increasing gradient of methanol from 0 to 10 % in 50 mM Tris-acetate (pH 7.5) (0 – 40 min : 0 – 10 %, 40 – 80 min : 10 %) at the flow rate of 0.70 ml/min. Elution positions of nucleotides were determined by measuring absorbance at 260 nm. Metaphosphate (Wako Pure Chemical Industries, Osaka, Japan) was routinely used at 1.0 mg/ml as poly(P), unless otherwise stated. One unit of enzyme activity was defined as 1.0 μmol of NADP or NADPH produced in 1 min at 37 ℃, and specific activity was expressed in units/mg protein. Protein concentration was determined by the method of Bradford (26) with bovine serum albumin as a standard.

Phosphatase activity of the enzyme was assayed by measuring orthophosphate released as described (27) in an above-described reaction mixture without NAD.

Amino acid sequence analysis. For internal amino acid sequence, the purified enzyme was digested with trypsin (Nacalai Tesque, Kyoto, Japan), loaded on TSKgel ODS-80Ts QA column (0.20 x 25 cm) (Tosoh, Tokyo, Japan) equipped with Waters 600E HPLC system (Millipore, Bedford, MA), and eluted with a linear gradient of acetonitrile in 0.10 % trifluoroacetic acid (0 – 90 %, 20 ml) into 200 μl portions every 1 min. The fractions containing peptides were collected and analyzed with SHIMAZU/PSQ-1 system (Shimazu, Kyoto, Japan). For N-terminal amino acid sequence, the purified enzyme was directly analyzed with Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA).

Purification of poly(P)/ATP-NAD kinase from *M. flavus*. Centrifugation was carried out at 20,000 x g, 4 ℃, for 20 min and dialysis was at 4 ℃ overnight against KND (10 mM potassium phosphate (pH 7.0), 0.10 mM NAD, and 0.50 mM dithiothreitol). The cells of *M. flavus* were pre-grown aerobically at 30 ℃ for 24 h in 650 ml of Luria-Bertani (LB) medium (28). The whole culture was used to inoculate a 65 l liquid medium (pH 7.2) consisting of 0.10 % $(NH_4)_2SO_4$, 0.05 % $MgSO_4 7H_2O$, 0.10 % KH_2PO_4, 0.40 % Na_2HPO_4, 0.50 % yeast extract, and 0.50 % glucose, and the cells were grown aerobically at 30 ℃ for 24 h. The cells (440 g wet wt.) were collected, suspended in 400 ml of KND, and treated with 0.20 mg/ml lysozyme at 37 ℃ for 20 min. After addition of 0.40 ml of 1.0 M phenylmethylsulfonyl fluoride, the cells were disrupted by Sonifire (Branson, Danbury, CT) and the resultant cell extract was fractionated with ammonium sulfate (20 – 40 %). The precipitate with both poly(P)- and ATP-NAD kinase activities was dissolved in 250 ml of KND, dialyzed, and then the solution was applied onto a DEAE-Toyopearl 650M column (4.2 x 48 cm) (Tosoh) equilibrated with KND. The kinases were eluted with a linear gradient of NaCl in KND (0 – 700 mM, 2,000 ml). The fractions with the two activities, which were obtained by elution with 450 - 500 mM NaCl, were combined, saturated with ammonium sulfate (15 %), and then directly applied onto a Butyl-Toyopearl 650M column (2.6 x 9.2 cm) (Tosoh) equilibrated with KND containing ammonium sulfate (15 %). The kinases were eluted with a linear gradient of ammonium sulfate in KND (15 – 0 %, 600 ml). The fractions with the two activities, which were obtained by elution with 9.0 – 6.0 % ammonium sulfate, were combined, dialyzed, and after supplementation with 10 mM $MgCl_2$, the dialysate was loaded onto an AF-Blue Toyopearl 650 ML column (0.80 x 20 cm) (Tosoh)

equilibrated with KNDMg (KND containing 10 mM $MgCl_2$). The kinases were eluted stepwise with 30 ml of KNDMg containing 1.0 M NaCl, 30 ml of KNDMg containing 2.0 M NaCl, and then with 30 ml of KNDMg containing 3.0 M NaCl. The fractions with the two activities, which were obtained by elution with 1.0 - 3.0 M NaCl, were combined, concentrated to about 3.0 ml by ultrafiltration with an Amicon model 8200 (Amicon, Beverly, MA), and then loaded onto a Sephacryl S-200 HR column (2.7 x 54 cm) (Amersham Pharmacia Biotech, Buckinghamshire, England) equilibrated with KND containing 150 mM NaCl. The enzymes were eluted with KND containing 150 mM NaCl into 3.0 ml fractions every 4.4 min, and the fractions containing two NAD kinase activities (fraction nos. 64 - 66) were combined, dialyzed, and used as a purified enzyme.

Other analytical methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (29). Native-PAGE was done as described (29) without SDS. Proteins in the gel were visualized by Coomassie Brilliant Blue R-250. Molecular mass of the enzyme was estimated by gel filtration chromatography on Sephacryl S-200 HR column (2.7 x 54 cm) with Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) as recommended by manufacturer. Activity staining of NAD kinase was performed as described (30).

RESULTS

Purification of poly(P)/ATP-NAD kinase from *M. flavus*. Both poly(P)- and ATP-dependent NAD kinases were purified approximately 4,000-5,000 folds by measuring poly(P)- and ATP-dependent NAD kinase activities, respectively, from cell extract of *M. flavus* with 6.0 – 8.0 % of activity yields (Table 1). The purified enzyme migrated as a single protein band on SDS-PAGE (34 kDa) (Fig. 1A) and native-PAGE (Fig. 1B, lane 1). By activity staining for NAD kinase, only one position, corresponding to that of the purified enzyme, was stained in the presence of poly(P) (Fig. 1B, lane 2) or ATP (Fig. 1B, lane 3), but no positions were stained in the absence of both poly(P) and ATP (Fig. 1B, lane 4). The protein corresponding to the stained band was extracted from the gel, analyzed with SDS-PAGE, and was revealed to be 34 kDa (data not shown). Furthermore, poly(P)- and ATP-dependent NAD kinase activities were co-eluted in a single peak in every chromatography (data not shown) and always recovered with an approximately constant activity ratio (Table 1). On the gel filtration chromatography on Sephacryl S-200 HR column, the two activities were

eluted in a single peak as a 68 kDa protein (data not shown). On the basis of these results described above, it was concluded that a single enzyme, a 68 kDa protein consisting of two identical 34 kDa subunits, was responsible for both poly(P)- and ATP-dependent NAD kinase activities. The 68 kDa protein was designated poly(P)/ATP-NAD kinase. N-terminal amino acid sequence of the purified enzyme was determined to be ^{1}PYTPGRRILVLTHTGRE DAISAALQATR^{28}. Internal amino acid sequences of the enzyme were also determined to be ^{1}FRLPTDGWRGPVTAQE^{16}, ^{1}ALFTRPLVVGPR^{12}, ^{1} GYNVPLLAVNLGHVGFLAES ER^{22}, ^{1}MALDVVVHVE^{10}, ^{1}TVQAIASESYVVIER^{15}, and ^{1}TWALNEASVEK^{11}.

TABLE 1 Purification of poly(P)/ATP-NAD kinase from *M. flavus*

Step	Total Protein (mg)	Poly(P)-dependent activity				ATP-dependent activity				Ratio (b/a)
		Total activity (units)	Yield (%)	Specific activity (a) (units/mg)	Purification (fold)	Total activity (units)	Yield (%)	Specific activity (b) (units/mg)	Purification (fold)	
Cell extract	23,562	23.2	100	0.00098	1.0	26.2	100	0.0011	1.0	1.1
AS[a] (20-40 %)	17,000	22.7	98	0.0013	1.3	22.7	86	0.0013	1.2	1.0
DEAE-Toyopearl	270	16.0	69	0.059	60.2	16.5	63	0.061	55.5	1.0
Butyl-Toyopearl	20.3	9.62	42	0.474	480	10.3	39	0.51	464	1.1
AF-Blue Toyopearl	3.00	4.93	21	1.64	1,684	5.23	20	1.74	1,591	1.1
Sephacryl S-200	0.35	1.85	8.0	5.29	5,378	1.73	6.6	4.94	4,500	0.9

[a] Ammonium sulfate

Properties of poly(P)/ATP-NAD kinase purified from *M. flavus*. Poly(P)/ATP-NAD kinase purified from *M. flavus* was characterized. The enzyme completely phosphorylated NAD to NADP utilizing poly(P) or ATP (data not shown). ATP and dATP were utilized more effectively than other nucleoside triphosphates (Table 2). Commercially available poly(P)s were utilized, except for trimetaphosphate [cyclic form of tripolyphosphate] (Table 2) and the kinetic constants of the enzyme showed that the enzyme preferred ATP to poly(P) [poly(P)4] (Table 3). *p*-Nitrophenylphosphate was not utilized (Table 2), indicating that NAD phosphorylation was not due to the reverse (phosphatase) reaction of the enzyme (31). The enzyme did not show NAD kinase activity that utilizes glucose-6-phosphate (Table 2), although such activity has been found in some coryneform bacteria isolated from sewage sludge (32). In the absence of NAD, no phosphatase activity for ATP and poly(P)4 was detected. The enzyme required bivalent metal ions such as Mg^{2+} and Mn^{2+} for their poly(P)- and ATP-dependent NAD kinase activities, and Mn^{2+} was more effective activator (Table 4).

Both poly(P)- and ATP-dependent NAD kinase activities of the enzyme were completely

inhibited by 1.0 mM HgCl2 or *p*-chloromercuribenzoate. Optimum pH and temperature were pH 7.0 in Tris-HCl and 55 ℃, respectively, irrespective of phosphoryl donors (Fig. 2A, B). Both poly(P)- and ATP-dependent NAD kinase activities were lost after incubation for 10 min at 60 ℃ (Fig. 2C). The enzyme was inhibited by NADPH and NADH, while not affected by NADP (Table 5).

The enzyme also showed poly(P)- and ATP-dependent NADH kinase activities that were confirmed with TLC (data not shown) and HPLC (Fig. 3) analyses. ATP-dependent NADH kinase activity was 65 % of that of ATP-dependent NAD kinase one, while poly(P)-dependent NADH kinase one was only 9.5 % to poly(P)-dependent NAD kinase one. Adenosine, ADP, and ADP-ribose were also phosphorylated with lower efficiencies (less than 10 % than NADH) in the presence of poly(P) that was detected by TLC (data not shown). ATP-dependent phosphorylations of these compounds were not definitely confirmed by TLC analysis (data not shown).

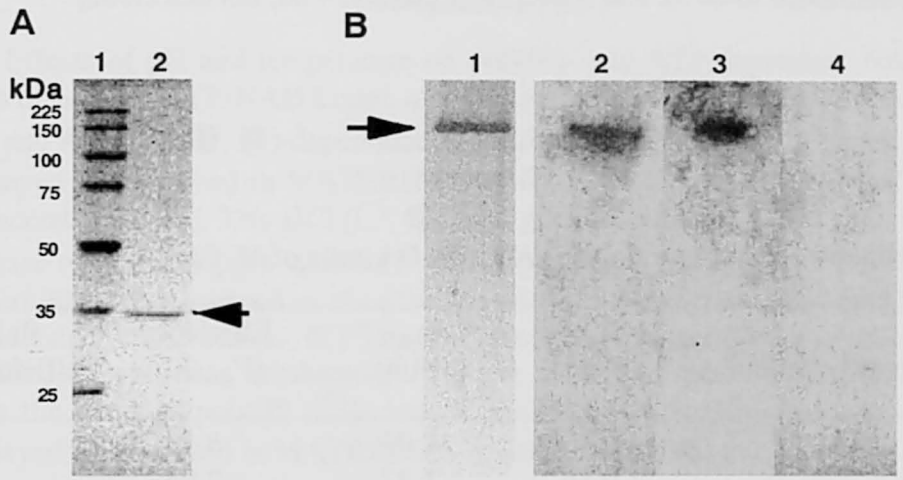

FIG. 1 PAGE of poly(P)/ATP-NAD kinase of *M. flavus*. (A) SDS-PAGE of poly(P)/ATP-NAD kinase purified from *M. flavus*. Lane 1: Protein markers (Novagen, Darmstadt, Germany). Lane 2: Purified enzyme (3.0 μg). (B) Activity staining for poly(P)/ATP-NAD kinase (5.0 μg) purified from *M. flavus* in the presence of poly(P) (lane 2), ATP (lane 3), and in the absence of both poly(P) and ATP (lane 4). Before activity staining, purified enzyme was electrophoresed on native-PAGE gel (lane 1). Arrows indicate the positions of the enzyme.

TABLE 2 Phosphoryl donor specificity of poly(P)/ATP-NAD kinase of *M. flavus*

Phosphoryl donor	Relative activity (%)	Phosphoryl donor	Relative activity (%)
ATP	100	Orthophosphate	nd
dATP	91	Pyrophosphate	nd
AMP	nd	Trimetaphosphate	nd
ADP	nd	Tripolyphosphate	40
GTP	88	Poly(P)4	151
CTP	73	Phosphate glass (type 35)	49
dTTP	74	Polyphosphate	138
UTP	87	Hexametaphosphate	88
Glucose-6-phosphate	nd	Metaphosphate	88
p-Nitrophenylphosphate	nd		

NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of the phosphoryl donors listed above. Activity for 5.0 mM ATP was relatively taken as 100 %. Metaphosphate, hexametaphosphate and polyphosphate (Wako Pure Chemical Industries) were used at 1.0 mg/ml. Others were from Sigma (St. Louis, MO) and used at 5.0 mM. A number of phosphoryl residues and molecular weight of the phosphate glass (type 35) are estimated to be 32 and 3,292, respectively. nd, not detected.

TABLE 3 Kinetic constants of poly(P)/ATP-NAD kinase of *M. flavus*

Compound	K_m (mM)	V_{max} (μM/h)	Efficiency (V_{max}/K_m)	Relative efficiency (%)
ATP	0.13	1.09	8.39	100
dATP	0.16	1.27	7.94	95
Poly(P)4	1.04	1.58	1.52	18
NAD (*+ATP*)[a]	0.83	1.76	1.76	20
NAD (*+poly(P)*)[b]	0.58	2.55	2.55	30

Kinetic constants were calculated with Lineweaver-Burk plot. 0.017 units (determined in the presence of 5.0 mM ATP) of poly(P)/ATP-NAD kinase of *M. flavus* was used. [a] Kinetic constants for NAD in the presence of 5.0 mM ATP. [b] Kinetic constants for NAD in the presence of 5.0 mM poly(P)4.

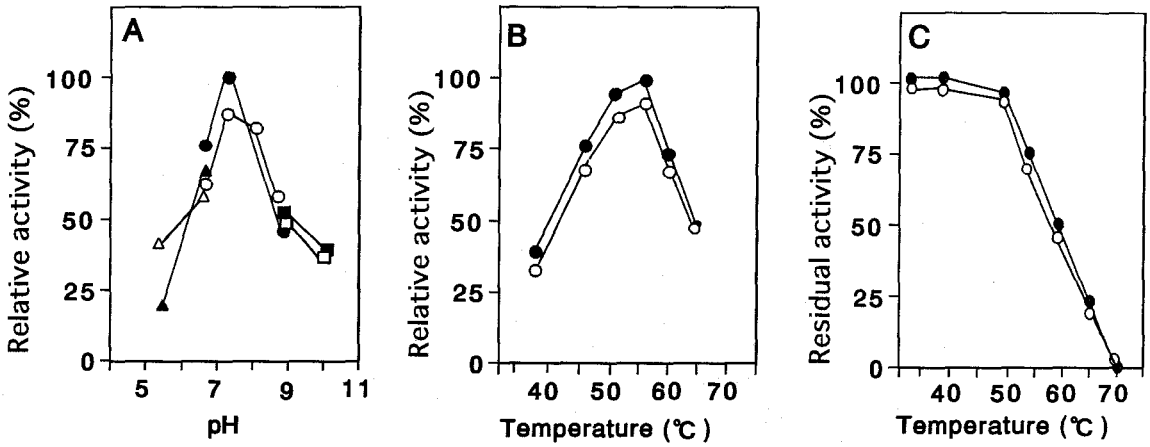

FIG. 2 Effects of pH and temperature on poly(P)- and ATP-dependent NAD kinase activities of poly(P)/ATP-NAD kinase of *M. flavus*. (A) Effects of pH on poly(P) (△, ○, □)- and ATP (▲, ●, ■)-dependent NAD kinase activities. NAD kinase activities were assayed as described in MATERIALS AND METHODS with each of 100 mM sodium acetate (△, ▲), Tris-HCl (○, ●), and glycine-NaOH (□, ■). (B) Effects of temperature on poly(P) (○)- and ATP (●)-dependent NAD kinase activities. NAD kinase activities were assayed as described in MATERIALS AND METHODS at each of the indicated temperatures. (C) Thermal stability of the poly(P)/ATP-NAD kinase. The purified enzyme was incubated for 10 min at each of the indicated temperatures and then the residual poly(P) (○)- and ATP (●)-dependent NAD kinase activities were assayed as described in MATERIALS AND METHODS.

TABLE 4 Effects of metal ions on poly(P)- and ATP dependent NAD kinase activities of poly(P)/ATP-NAD kinase of *M. flavus*

Metal	Poly(P)-dependent activity (%)	ATP-dependent activity (%)
None	nd	nd
$MgCl_2$	100	100
$MnCl_2$	143	136
$ZnCl_2$	30	51
$CaCl_2$	65	61
$CoCl_2$	51	28
$FeCl_2$	nd	nd
$CuCl_2$	nd	nd
NaCl	nd	nd
KCl	nd	nd
LiCl	nd	nd

Poly(P)- and ATP-dependent NAD kinase activities were assayed in the reaction mixture described in MATERIALS AND METHODS, in which 5.0 mM $MgCl_2$ was replaced for each of 1.0 mM metal ions listed above. Each of poly(P)- and ATP-dependent NAD kinase activities in the presence of 1.0 mM $MgCl_2$ was relatively taken as 100 %. nd., not detected.

TABLE 5 Effects of NADP, NADH, and NADPH on poly(P)- and ATP-dependent NAD kinase activities of poly(P)/ATP-NAD kinase of *M. flavus*

Compound	Conc. (mM)	Poly(P)-dependent activity (%)	ATP-dependent activity (%)
None	0	100	100
NADPH	0.02	100	100
	0.05	50	66
NADH	0.05	100	100
	0.50	50	66
NADP	0.50	100	100

Poly(P)- and ATP-dependent NAD kinase activities were assayed in the reaction mixture described in MATERIALS AND METHODS containing each of NADP, NADH, and NADPH at the indicated concentrations. Each of poly(P)- and ATP-dependent NAD kinase activities in the absence of NADP, NADH, and NADPH was relatively taken as 100 %.

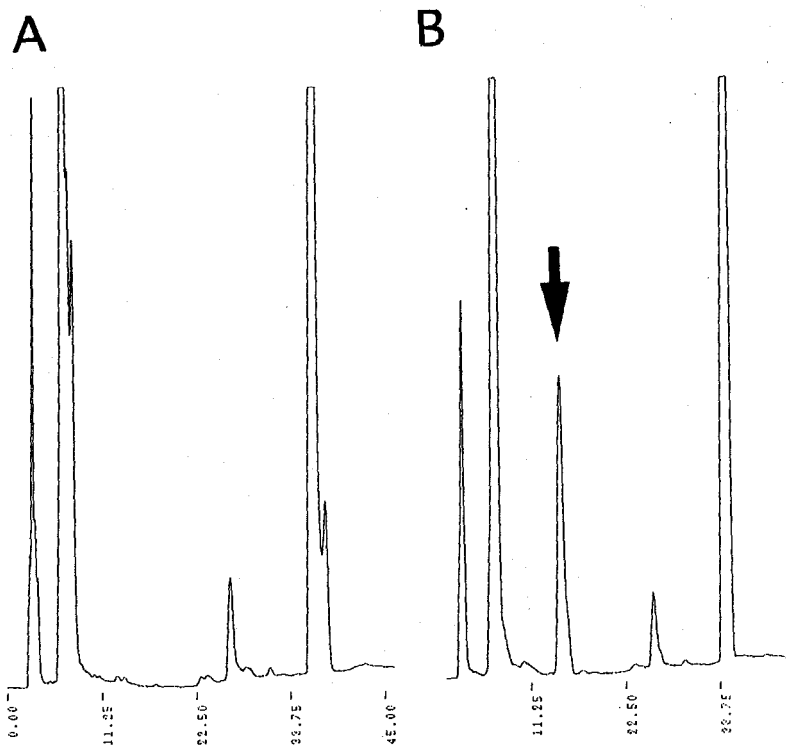

FIG. 3 Assay of NADH kinase activity. ATP-dependent NADH kinase reactions were performed for 30 min as described in MATERIALS AND METHODS in the absence (A) and presence (B) of NAD kinase (Mfnk). Arrow indicates the formed NADPH.

DISCUSSION

In this chapter, a novel enzyme was isolated from *M. flavus* for the first time that catalyzes the phosphorylation of NAD by the use of not only ATP, but also poly(P). The enzyme was designated "poly(P)/ATP-NAD kinase" to distinguish it from ATP-NAD kinase that utilizes ATP, but not poly(P). Among poly(P)-metabolizing enzymes, the isolation of poly(P)/ATP-NAD kinase is the fourth one succeeding poly(P) kinase (8-10), poly(P) phosphatase (15-18), and poly(P)/ATP-glucokinase (23, 24).

Existence of poly(P)/ATP-NAD kinase described in this chapter emphasizes a role of poly(P) as an ATP substitute. Previously Murata *et al.* observed that microorganisms having poly(P)-dependent NAD kinase activities also exhibit poly(P)-dependent glucokinase activities (6, 33). Poly(P)/ATP-glucokinase was also isolated from *M. flavus* (34). These facts of the co-occurrence of poly(P)-dependent NAD kinase and poly(P)-dependent glucokinase may imply some physiological consequence concerning metabolic activity of microorganisms. Briefly, for example, phosphogluconate pathway beginning with an intermediate glucose-6-phosphate is known as a main route for generation of reductant NADPH required for the biosynthesis of long-chain fatty acids and steroids (35). Therefore, it is likely that the abilities to utilize poly(P) for phosphorylation of NAD and glucose give much energetical advantages to produce NADP and glucose-6-phosphate, and thus, to supply NADPH physiologically via phosphogluconate pathway. Furthermore, poly(P)- and ATP-dependent NADH kinase activities of poly(P)/ATP-NAD kinase were found, although poly(P)- dependent NADH kinase activity was lower than ATP-dependent NADH kinase one. Although NADH-specific kinase was only partially purified from mitochondrial fraction of *S. cerevisiae* (36), no one has reported that NAD kinase showed NADH kinase activity. This finding likely seem to indicate that in *M. flavus* NADPH is formed through direct phosphorylation of NADH by poly(P)/ATP-NAD kinase, that is a distinct pathway from phosphogluconate one.

From a standpoint of structural biology, isolation of poly(P)/ATP-NAD kinase presents important problems, (i) what is the mechanism of the enzyme to utilize poly(P); (ii) why and how the enzyme acquires the ability to utilize poly(P) or ATP-NAD kinase loses the ability.

Isolation of poly(P)/ATP-NAD kinase also raises an another simple question where ATP-NAD kinase is ? Indeed some "NAD kinases" have been purified completely from pigeon liver (2), pigeon heart (3), *S. cerevisiae* (4), and *C. utilis* (5), but the possibility could not be

denied that they are poly(P)/ATP-NAD kinases, since attentions were not paid to poly(P)-dependent NAD kinase activities of these enzymes in their purification procedures (2-5). In order to identify NAD kinase as ATP-NAD kinase, attention to poly(P)-dependent NAD kinase activity of the NAD kinase would be required.

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Section 2 Molecular cloning of inorganic polyphosphate/ATP-NAD kinase gene from *Micrococcus flavus*

In the previous Section, inorganic polyphosphate [poly(P)]/ATP-NAD kinase was isolated that is discriminated from "ATP-NAD kinase" that utilizes ATP, not poly(P), for the phosphorylation of NAD. Isolation of poly(P)/ATP-NAD kinase postulates important problems, (i) what is the mechanism of the enzyme to utilize poly(P); (ii) why and how the enzyme acquires the ability to utilize poly(P) or ATP-NAD kinase loses the ability.

In order to address to these problems, structural analysis of the enzyme is indispensably required. However, no information as to the structure of NAD kinase was obtained, since no one had succeeded yet in molecular cloning of poly(P)/ATP-NAD kinase gene as well as ATP-NAD kinase gene (1). As the first step for the structural analysis of poly(P)/ATP-NAD kinase, molecular cloning of poly(P)/ATP-NAD kinase gene was undertaken and is described in this Section.

MATERIALS AND METHODS

Strains. *M. flavus* (IFO 3242) was cultured at 30 ℃ for 24 h in 300 ml liquid medium (pH 7.2) consisting of 0.10 % (NH4)2SO4, 0.05 % MgSO47H2O, 0.10 % KH2PO4, 0.40 % Na2HPO4, 0.50 % yeast extract, and 0.50 % glucose. Cultivation conditions of the derivative strains of *Escherichia coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany) were described in the text. As a host for plasmid amplification, *E. coli* DH5α (Toyobo, Osaka, Japan) was routinely cultured at 37 ℃ in Luria-Bertani (LB) medium (2) with ampicillin (100 μg/ml).

Assays. Poly(P)- and ATP-dependent NAD kinase activities were assayed as described (3) and protein concentrations were as given by Bradford (4). Details for these assays are indicated in Section 1 of this Chapter.

DNA sequence analysis. DNA sequence was determined using an automated DNA sequencer (Model 377; Applied Biosystems Division of Perkin-Elmer).

Construction of genomic DNA library. Genomic DNA of *M. flavus* was isolated with a standard method as described (2), partially digested with *Sau*3AI, and separated on 0.80 % agarose gel electrophoresis. The partially digested DNA fragments (4.0 – 8.0 kb) were

isolated with gene clean kit (Bio 101, Vista, CA) and ligated to pUC118 (Takara Biomedicals, Kyoto, Japan) digested with *Bam*HI, yielding recombinant DNA clones. Cells of *E. coli* DH5α were transformed with these DNA clones and then used as genomic DNA library of *M. flavus*.

Cloning of poly(P)/ATP-NAD kinase gene (*mfnk*). Genomic DNA library of *M. flavus* was screened by using mixtures of [^{32}P]-labeled oligonucleotides [5' AC(A/C/G/T)TGGGC (A/C/G/T)(C/T)T(G/C)AA(C/T)GA(A/G)GC 3'] which was designed on the basis of an internal amino acid sequence [TWALNEA] of poly(P)/ATP-NAD kinase purified from *M. flavus*. Poly(P)/ATP-NAD kinase gene (*mfnk*) was identified through a sequence analysis of DNA fragments derived from positive clones as described in RESULTS. *mfnk* was then amplified with PCR (Takara Biomedicals) from genomic DNA of *M. flavus* in a reaction mixture (100 μl) containing 2.5 U KOD polymerase (Toyobo), 0.25 μg genomic DNA, 40 pmol *Nde*I primer 5' GACATATGCCCTACACCCCCGGACGTC 3', 40 pmol *Hin*dIII primer 5' TGAAGCTTCA ATGCGTCCTCCCCGGGGTG 3', 20 nmol dNTPs, 100 nmol MgCl2, 8.0 % dimethyl sulfoxide, and reaction buffer #1 supplied with KOD polymerase. Cycle condition for PCR was as follows: 94 ℃ 30 sec, 50 ℃ 30 sec, 74 ℃ 30 sec, 30 cycles. A 1.1 kb PCR product, of which the nucleotide sequence was determined and identified to be *mfnk*, was isolated with gene clean kit as above, digested with *Nde*I and *Hin*dIII, and ligated into pET-17b (Novagen) digested with *Nde*I and *Hin*dIII, yielding pSK122. Derivative strains, SK122 and SK45 were obtained by transforming *E. coli* BL21(DE3)pLysS with pSK122 and pET-17b, respectively.

Expression of poly(P)/ATP-NAD kinase (Mfnk) in *E. coli*. For the expression of poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus*, SK122 was inoculated into 400 ml LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chroramphenicol, and cultured at 37 ℃ aerobically until A_{600} was 1.4. This culture was transferred into 13.5 l of the same medium with same antibiotics and cultivation was continued at 37 ℃ aerobically for 2 h until the A_{600} reached 0.70; then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.40 mM, and the cultivation was continued further at 18 ℃ aerobically for 3 days. As a control, SK45 in 10 ml medium was also treated with the same manner.

Purification of poly(P)/ATP-NAD kinase (Mfnk) expressed in *E. coli*. Centrifugation was carried out at 20,000 x g, 4 ℃ for 20 min, and dialysis was at 4 ℃ overnight against KNDE (KND containing 1.0 mM EDTA), unless otherwise stated. The SK122 cells (75 g

wet wt.) expressing Mfnk were collected, suspended in 100 ml of KNDE, and then disrupted with Sonifire (Branson, Danbury, CT). Cell extract after centrifugation was supplemented with 1.0 mM phenylmethylsulfonyl fluoride and Mfnk was precipitated with ammonium sulfate (25 – 30 %). The precipitate was dissolved in KNDE, dialyzed, applied onto a DEAE-Toyopearl 650M column (2.6 x 35 cm) equilibrated with KNDE, and proteins were eluted with a linear gradient of NaCl (0 – 600 mM, 600 ml) in 13 ml fractions every 8.0 min. Fractions with Mfnk, which were obtained by elution with 400 - 450 mM NaCl, were combined, and after saturation with ammonium sulfate (15 %), applied onto a Butyl-Toyopearl 650M column (2.8 x 8.0 cm) equilibrated with KNDE containing ammonium sulfate (15 %). Proteins were eluted with a linear gradient of ammonium sulfate (15 – 0 %, 180 ml) in 6.0 ml fractions every 6.0 min. The fractions with Mfnk, which were obtained by elution with 5.0 – 0 % ammonium sulfate, were combined, dialyzed against KNDE containing 10 mM MgCl2, and loaded onto an AF-Blue Toyopearl 650 ML column (1.8 x 9.6 cm) equilibrated with KNDE containing 10 mM MgCl2. Proteins were eluted with a linear gradient of NaCl (0 – 500 mM, 200 ml) and then 500 mM NaCl (50 ml) in 2.0 ml fractions every 2.0 min. Fractions with Ppnk, which were obtained by elution with 500 mM NaCl (fraction nos. 20 - 28), were pooled and dialyzed. The dialysate was loaded onto a ResourceQ column (6.4 x 30 mm) (Amersham Pharmacia Biotech) equilibrated with KNDE, and eluted with a linear gradient of NaCl (0 - 600 mM, 600 ml) in 500 μl fractions every 15 s. Fractions with Mfnk, which were obtained by elution with 540 – 560 mM NaCl, were combined, dialyzed, and used as a purified Mfnk.

Other methods. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (5). Blast (6) on the World-Wide Web site of the DNA Databank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html) was used for the homology search. Sequence data were obtained from the GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) and SwissProt (http://www.genome.ad.jp/dbget-bin/www_ bfind?swissprot-today) databases.

Accession number. Nucleotide sequence of *Sma*I - *Pst*I region (2,497 b) determined in this study has been deposited in GenBank/EMBL/DDBJ under accession number AB070351.

RESULTS AND DISCUSSIONS

Cloning of poly(P)/ATP-NAD kinase gene (*mfnk*). As a consequence of the screening of *M. flavus* genomic DNA library (about 25,000 colonies), two positive clones were obtained. Nucleotide sequence analysis of DNA fragments derived from these positive clones revealed that *Sma*I - *Pst*I region (2,497 b) in these DNA fragments contained three open reading frames (orf-1, orf-2, and orf-3) (Fig. 1A). Among them, deduced polypeptide from orf-2 showed identities with N-teminal and internal amino acid sequences of poly(P)/ATP-NAD kinase purified from *M. flavus*, indicating that orf-2 encodes poly(P)/ATP-NAD kinase. The orf-2 consisting of 1,089 b nucleotides, was named *mfnk* (Fig. 2). A possible ribosome binding site (AAGGA) was observed three nucleotides upstream of ATG initiation codon of *mfnk*, but no typical promotor region was found. Then, *mfnk* was cloned and expressed in SK122 as described in MATERIALS AND METHODS. Cell extract of SK122 showed high activities (0.44 units/mg) of poly(P)- and ATP-dependent NAD kinases (Table 1), that were extremely higher than those (0 and 0.0042 units/mg, respectively) of cell extracts of control strain, SK45. This expression study indicates that *mfnk* is surely encodes poly(P)/ATP-NAD kinase (Mfnk). This cloning was the first one of the gene encoding poly(P)/ATP-NAD kinase as well as ATP-NAD kinase. Features of primary structure of Mfnk are discussed in Section 3 of Chapter III.

Nucleotide sequence analysis also showed the possibility that *mfnk* has relation with orf-3, since TGA termination codon of *mfnk* shares nucleotides with a putative ATG initiation codon of orf-3 (Fig. 1B). A possible Shine-Dalgarno sequence (AGGA) was also observed three nucleotides upstream of the putative ATG initiation codon of orf-3 (Fig. 1B). The deduced amino acid sequence from orf-3 showed significant homologies with DNA repair protein (RecN), such as RecN of *E. coli* (SwissProt: RECN ECOLI) (42 % homology) and RecN of *Bacillus subtilis* (RECN BACSU) (40 %). Deduced polypeptide from orf-1 exhibited homologies with hemolysin of *Treponema hyodysenteriae* (HLYA TREHY) (51 %) and the proteins belonging to methyltransferase superfamily, such as rRNA methyltransferase of *Pseudomonas aeruginaosa* (RRMJ PSEAE) (42 %). Hemolysin is known as an exotoxin that attacks blood cell membranes and causes cell rupture (7). On the contrary to the case of orf-3, orf-1 was supposed to have no relation with *mfnk*, since orf-1 was located 116 nucleotides upstream from *mfnk*.

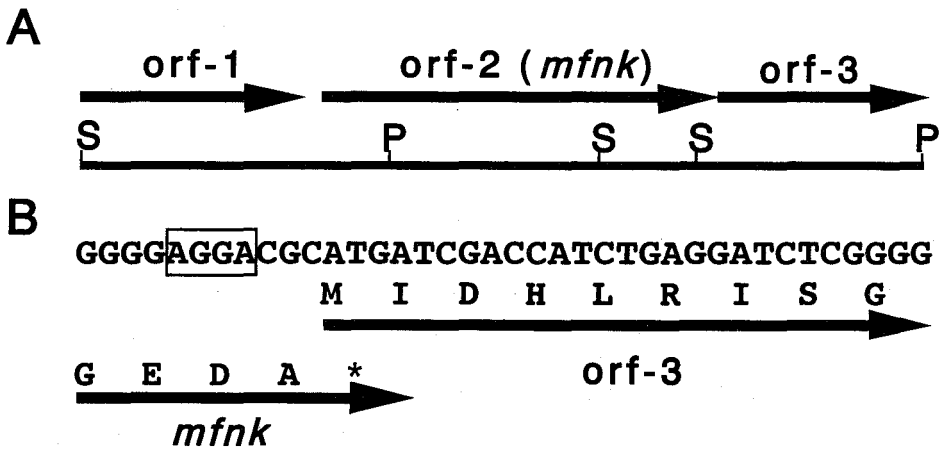

FIG. 1 Nucleotide sequence of 2,497 b *Sma*I and *Pst*I region. (A) Restriction enzyme map of the 2,497 b *Sma*I - *Pst*I region. Three open reading frames were indicated by bold arrows. Abbreviations; S, *Sma*I; P, *Pst*I. (B) Nucleotide sequence of 3' -terminal region of *mfnk*. Deduced peptides from *mfnk* and orf-3 are indicated by bold arrows and translations into amino acids. Putative ribosome binding site of orf-3 was boxed.

Purification of poly(P)/ATP-NAD kinase (Mfnk) expressed in *E. coli*. From cell extract of SK122 expressing poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus*, Mfnk was purified as described in MATERIALS AND METHODS (Table 1). As a result of SDS-PAGE (Fig. 3) and Sephacryl 200SH (data not shown) analyses, purified Mfnk was shown to be a dimer consisting of 34 kDa subunits. N-terminal amino acid sequence of Mfnk was determined to be PYTPGRIV. These properties of Mfnk were identical with those of poly(P)/ATP-NAD kinase purified from *M. flavus*.

TABLE 1 Purification of Mfnk from SK122

		Poly(P)-dependent activity				ATP-dependent activity				
Step	Total Protein (mg)	Total activity (units)	Yield (%)	Specific activity (a) (units/mg)	Purification (fold)	Total activity (units)	Yield (%)	Specific activity (b) (units/mg)	Purification	Ratio (b/a) (fold)
Cell extract	5,633	2,490	100	0.44	1.0	2,490	100	0.44	1.0	1.0
AS[a] (25-30 %)	1,415	2,346	94	1.72	3.9	2,240	90	1.58	3.6	1.0
DEAE-Toyopearl	390	1,440	58	3.70	8.4	1,428	57	3.66	8.3	1.0
Butyl-Toyopearl	120	1,296	52	10.8	25	1,228	51	10.2	24	1.0
AF-Blue Toyopearl	50.0	500	20	10.0	23	484	19	9.68	22	1.0
Resource Q	10.5	255	10	24.3	55	244	9.8	22.9	52	1.0

[a] Ammonium sulfate

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        10         20         30         40         50         60         70         80
ATGCCCTACACCCCCGGACGTCGCATCCTCGTCCTGACCCACACGGGCCGCGAGGACGCCATCAGCGCGGCGCTGCAGGC
M  P  Y  T  P  G  R  R  I  L  V  L  T  H  T  G  R  E  D  A  I  S  A  A  L  Q  A
        90        100        110        120        130        140        150        160
CACCCGCATGTTCGCGGAGGAGGGCCTGGTCACCGTCATGCTCGAACAGGACGTGGCCGCCATCCGCGCGGCCGCGGGGG
 T  R  M  F  A  E  E  G  L  V  T  V  M  L  E  Q  D  V  A  A  I  R  A  A  A  G  D
       170        180        190        200        210        220        230        240
ACCCCCCGGAGTTCGCGCCGGAGACCCTCGGGGTCGACTGCGAGCTCGAGGACATCACCCTCGGCCTCGTCCTCGGCGGC
  P  P  E  F  A  P  E  T  L  G  V  D  C  E  L  E  D  I  T  L  G  L  V  L  G  G
       250        260        270        280        290        300        310        320
GACGGCTCGGTGCTGCGGGCGGCCGACTTCGTCCGCGGCTACAACGTGCCGCTGCTGGCGGTGAACCTGGGCCACGTCGG
D  G  S  V  L  R  A  A  D  F  V  R  G  Y  N  V  P  L  L  A  V  N  L  G  H  V  G
       330        340        350        360        370        380        390        400
CTTCCTCGCCGAGTCCGAGCGCACCGACCTGCACCGCACCGTGCAGGCGATCGCCTCGGAGTCCTACGTGGTGATCGAGC
 F  L  A  E  S  E  R  T  D  L  H  R  T  V  Q  A  I  A  S  E  S  Y  V  V  I  E  R
       410        420        430        440        450        460        470        480
GCATGGCCCTGGACGTCGTCGTGCACGTGGAGGGCCGCGAGGTGGCCCGCACGTGGGCCCTCAACGAGGCCTCCGTGGAG
  M  A  L  D  V  V  V  H  V  E  G  R  E  V  A  R  T  W  A  L  N  E  A  S  V  E
       490        500        510        520        530        540        550        560
AAGTCCCACCGGGAGCGGATGCTCGAGGTGGTCGTCTCCGTGGACAACTCGCCGCTGACGTCCTTCGGCTGCGACGGCGT
K  S  H  R  E  R  M  L  E  V  V  V  S  V  D  N  S  P  L  T  S  F  G  C  D  G  V
       570        580        590        600        610        620        630        640
CGTGCTGGCCACCCCCACCGGCTCCACGGCCTACGCCTTCTCCGCCGGGGGCCCCGTGGTGTGGCCCTCCGTGGAGGCCC
 V  L  A  T  P  T  G  S  T  A  Y  A  F  S  A  G  G  P  V  V  W  P  S  V  E  A  L
       650        660        670        680        690        700        710        720
TGCTGTGCGTGCCCATCAGCGCCCACGCCCTGTTCACCCGCCCCCTcGTGGTGGGTCCGCGCTCCACGATCGGtGtGGAC
  L  C  V  P  I  S  A  H  A  L  F  T  R  P  L  V  V  G  P  R  S  T  I  G  V  D
       730        740        750        760        770        780        790        800
GTGCTCACCCGCACCCGCGAGACCGGTGTGCTGTGGTGTGACGGCCGGCGCACCGTCGAACTGCCACCGCAGGCCCGGGT
V  L  T  R  T  R  E  T  G  V  L  W  C  D  G  R  R  T  V  E  L  P  P  Q  A  R  V
       810        820        830        840        850        860        870        880
CGAGGTGTCCCGGTCGGcAGAGCCGGtGCGCCTGGcCCGTCTCAACCCGACTCCCTTCGcGGAACGGcTGGTGCGCAAGT
 E  V  S  R  S  A  E  P  V  R  L  A  R  L  N  P  T  P  F  A  E  R  L  V  R  K  F
       890        900        910        920        930        940        950        960
TcCGGCTGCCCACGGACGGcTGGCGCGGCCCCGTCACCGcCCAAGAGCGGGCGGGCGTGCTCCACGAGGTGGAGACCGTC
  R  L  P  T  D  G  W  R  G  P  V  T  A  Q  E  R  A  G  V  L  H  E  V  E  T  V
       970        980        990       1000       1010       1020       1030       1040
GAGCCGCGGCACgcGGGGCCgcGTCCCGACGTCGTCGAACCGGCCACCATGCCGCTGTCGGTGCCCTCGCCCGCGGACAT
E  P  R  H  A  G  P  R  P  D  V  V  E  P  A  T  M  P  L  S  V  P  S  P  A  D  I
      1050       1060       1070       1080
CCAACGCCACCGCGACCGCGGGGCGCGTCACCCCGGGGAGGACGCATGA
 Q  R  H  R  D  R  G  A  R  H  P  G  E  D  A  *
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FIG. 2 Nucleotide sequence of *mfnk*. Deduced amino acid sequence of Mfnk is indicated below nucleotide sequence.

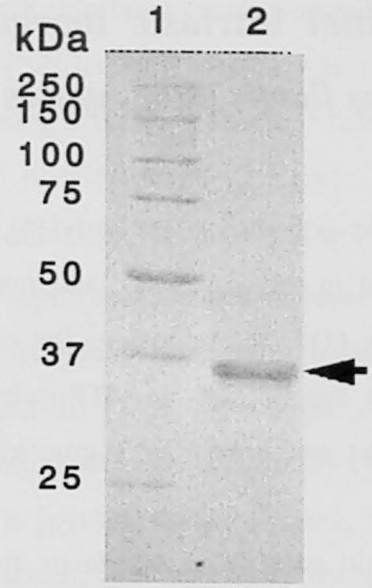

FIG. 3 SDS-PAGE of poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus* purified from SK122. Lane 1: Protein markers (Bio-Rad, Hercules, CA). Lane 2: Purified enzyme (5.0 μg). Arrow indicates the position of poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus* purified from SK122.

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Section 3 Evidence that intrinsic inorganic polyphosphate of *Micrococcus flavus* participates in NADP synthesis

In *Escherichia coli*, inorganic polyphosphate [poly(P)] is supposed to function as a regulator for stress and survival in this organism, and not as an ATP substitute for the phosphorylation of biomolecules (1). In contrast to the case of *E. coli*, in *Micrococcus flavus*, poly(P) is presumed to function as an ATP substitute through the actions of poly(P)/ATP-NAD kinase (Mfnk) and poly(P)/ATP-glucokinase as described in previous Sections.

In order to show that intrinsic poly(P) of *M. flavus* functions as an ATP substitute, intrinsic poly(P) was isolated from this organism and shown to be utilized by Mfnk as phosphoryl donors. Results are described in this Section.

MATERIALS AND METHODS

Isolation of intrinsic [^{32}P]-labeled poly(P). *M. flavus* (IFO 3242) was aerobically cultured in 10 ml of Luria-Bertani (LB) medium (2) containing 440 pmol of [^{32}P] orthophosphate (18.2 μCi) (ICN Biochemicals, Costa Mesa, CA) at 30 ℃ for 24 h. Intrinsic [^{32}P]-labeled poly(P) was isolated from the harvested cells as described (3) with slight modifications as follows: Cells were suspended in 500 μl GTC solution [4.0 M guanidine thiocyanate and 50 mM Tris-HCl (pH 7.5)] and sonicated by Sonifier (Branson, Danbury, CT) for 1.0 min. To the resulting cell extract, 500 μl of 95 % ethanol, 30 μl of 10 % sodium dodecyl sulfate (SDS), and 20 μl of glassmilk supplied with gene clean kit (Bio 101, Vista, CA) were added. After mixing and keeping the solution for 5.0 min, the glassmilk was washed with New Wash solution supplied with gene clean kit. Then the glassmilk was suspended in 50 mM Tris-HCl (pH 7.5) containing 5.0 mM MgCl2, 10 μg DNase, and 10 μg RNase, and incubated at 37 ℃ for 20 min. After washing glassmilk by GTC solution, 95 % ethanol, and New Wash solution, the intrinsic poly(P) was eluted with 40 μl of 50 mM Tris-HCl (pH 7.5) by boiling for 2 min.

Purification of poly(P)/ATP-NAD kinase (Mfnk). Poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus* was expressed and purified from SK122 as described in Section 2 of this Chapter.

Analysis of intrinsic [^{32}P]-labeled poly(P). The intrinsic [^{32}P]-labeled poly(P) isolated

from *M. flavus* was analyzed with thin layer chromatography (TLC) as follows. Instead of authentic poly(P) or ATP, the [^{32}P]-labeled poly(P) was used as phosphoryl donor in the reaction mixture (20 μl) containing 5.0 mM NAD, 5.0 mM MgCl2, 100 mM Tris-HCl (pH 7.0), [^{32}P]-labeled poly(P), and purified Mfnk (2.0 μg). The reaction mixture was then incubated at 37 ℃. At the prescribed time, 1.0 μl of the reaction mixture was withdrawn, spotted on a silica gel, and developed with various solvents [solvent I: isobutyrate – 500 mM NH4OH (5:3 v/v), solvent II: saturated ammonium sulfate – 100 mM sodium acetate - isopropanol (40:10:1 v/v), solvent III: 1.0 M ammonium acetate - 99.5 % ethanol (3:7 v/v)]. These solvents definitely separate nucleotide compounds (4). As controls, authentic NAD, NADP, orthophosphate, poly(P)s [pyrophosphate (Sigma, St. Louis, MO), tripolyphosphate (Sigma), tetrapolyphosphate (Sigma), and metaphosphate (Wako Pure Chemical Industries, Osaka, Japan)], and nucleoside triphosphates (ATP, GTP, CTP, TTP, and UTP) (Sigma) were also developed. The developed [^{32}P]-labeled compounds were visualized with an Imaging plate type BAS-III (Fuji Photo Film, Tokyo, Japan) and the nucleotides were with exposure to UV light. Orthophosphate and poly(P) were detected as described (5). To confirm the formation of [^{32}P]-labeled NADP, the 2 h-incubated reaction mixture was further incubated at 37 ℃ for 30 min in the presence of glucose-6-phospahte dehydrogenase (0.10 units) (Sigma) and 0.50 mM glucose-6-phospahte. Then, the formation of [^{32}P]-labeled NADPH was checked with TLC analysis.

Intrinsic [^{32}P]-labeled poly(P) and Mfnk activities at various growth phases. The cells of *M. flavus* were cultured in 10 ml of LB media with and without 440 pmol of [^{32}P] orthophosphate (18.2 μCi) at 30 ℃. At 6, 12, 24, and 40 h cultivation times, from both cultures the cells (about 1.0 x 10^8 cells), which were estimated from A_{600} of the cultures, were collected and the viable cell number of each culture was counted by plating the diluted cultures on the LB medium solidified with 1.5 % agarose. From the cells collected, intrinsic [^{32}P]-labeled poly(P) and cell extract were prepared as described above. The [^{32}P]-labeled poly(P) was incubated with Mfnk and analyzed as described above. In order to calculate the amount of [^{32}P]-labeled NADP formed through the reaction of Mfnk, silica gels on the positions corresponding to those of [^{32}P]-labeled NADP were recovered and analyzed by scintillation counter (LS6500, Beckman Instruments, Fullerton, CA) with authentic [^{32}P] orthophosphate as a control. The amount of [^{32}P]-labeled poly(P) was expressed as that of [^{32}P]-labeled NADP formed. Poly(P)-dependent NAD kinase activity of the cell extract was assayed as described (4) with authentic poly(P) (metaphosphate) as phosphoryl donor and

protein concentrations were as described by Bradford (6). Details for these assays are described in Section 1 of this Chapter.

RESULTS AND DISCUSSION

Isolation and analysis of intrinsic poly(P) of *M. flavus*. From *M. flavus* cells cultured for 24 h (stationary phase : A_{600} = 8.0) in LB medium containing [^{32}P] orthophosphate, intrinsic [^{32}P]-labeled poly(P) was isolated and its phosphoryl donor activity for Mfnk was investigated as described in MATERIALS AND METHODS. As shown in Fig. 1, the reaction mixture at 0 min contained no [^{32}P]-labeled nucleotides, but only included the [^{32}P]-labeled compounds that remained on the origins. The [^{32}P]-labeled compounds were supposed to be [^{32}P]-labeled poly(P), since authentic poly(P) tested also remained on the origins when developed with solvents I and III (data not shown). In fact, as the Mfnk reaction proceeded, spots on the positions corresponding to authentic NADP appeared (Fig. 1), suggesting that [^{32}P]-labeled poly(P) was utilized by Mfnk for the phosphorylation of NAD and [^{32}P]-labeled NADP was consequently formed. The formation of [^{32}P]-labeled NADP was confirmed to have a biological activity by the use of glucose-6-phosphate and glucose-6-phosphate dehydrogenase as described in MATERIALS AND METHODS. From these results described above, it was suggested that intrinsic poly(P) functions as a phosphoryl donor for the phosphorylation of NAD in *M. flavus*.

Poly(P) and Mfnk activities at various growth phases of *M. flavus*. The amount of intrinsic [^{32}P]-labeled poly(P), poly(P)-dependent NAD kinase activities, and viable cell numbers at various growth phases of *M. flavus* were determined (Fig. 2A, B). The poly(P), that could be utilized by Mfnk, was isolated from the cells at early (Fig. 2A, B, 12 h cultivation time) and mid stationary (Fig. 2A, B, 24 h cultivation time) phases, but not from those at log (Fig. 2A, B, 6 h cultivation time) and late stationary (Fig. 2A, B, 40 h cultivation time) phases. Numbers of viable cells at late stationary phase were approximately equal to those at mid stationary phase (Fig. 2B). The extracts prepared from the cells collected at these phases showed the same degrees of poly(P)-dependent NAD kinase activities (Fig. 2B). Thus, it was indicated that in *M. flavus* intrinsic poly(P) was utilized by poly(P)-metabolizing enzymes including Mfnk during the stationary phase.

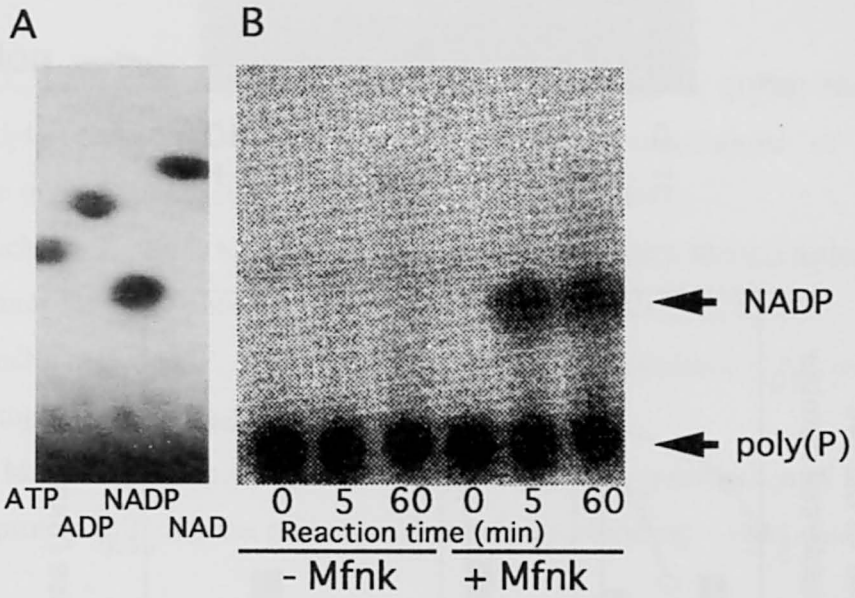

FIG. 1 Analyses of intrinsic [^{32}P]-labeled poly(P) of *M. flavus*. Developed compounds were visualized by UV light (A) or Imaging plate type BAS III (B). Phosphoryl donor activity of [^{32}P]-labeled poly(P) was investigated as described in MATERIALS AND METHODS in the presence (+Mfnk) and absence (-Mfnk) of Mfnk. Solvent I was used for the development. Positions corresponding to those of poly(P) and NADP are indicated by arrows. Other compounds tested (GTP, CTP, TTP, UTP, NADPH, and orthophosphate) were well developed by solvent I, although their positions are not presented here.

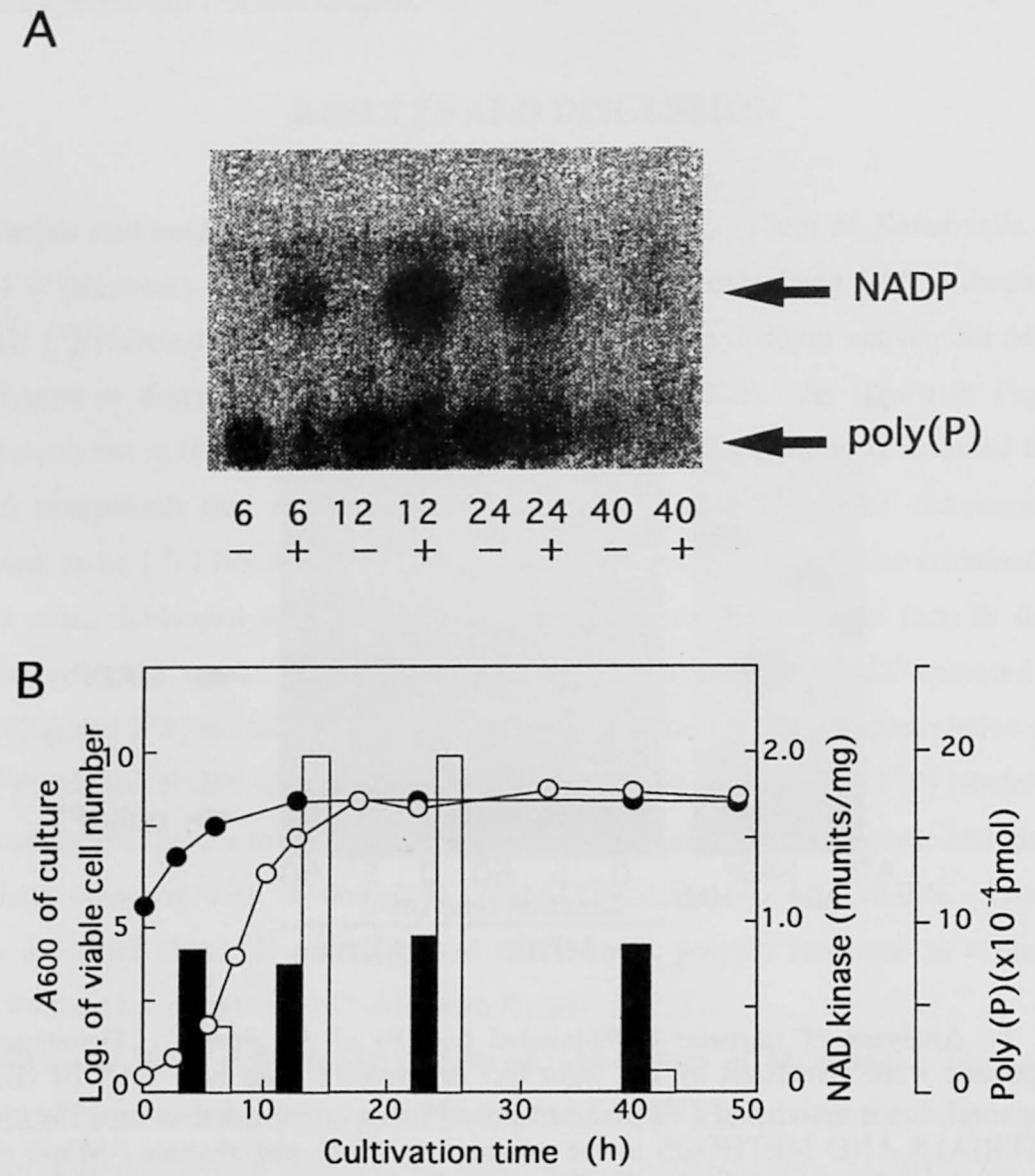

FIG. 2 Intrinsic [^{32}P]-labeled poly(P) and Mfnk activities at various growth phases of *M. flavus*. (A) TLC analysis of [^{32}P]-labeled poly(P) isolated from the cells of *M. flavus* at various growth phases. The intrinsic [^{32}P]-labeled poly(P)s incubated with Mfnk were analyzed with TLC as described in MATERIALS AND METHODS. Absence and presence of Mfnk are indicated by - and + , respectively. Numbers represent cultivation times at which the cells were collected to isolate [^{32}P]-labeled poly(P). Solvent I was used for the development. The positions corresponding to poly(P) and NADP were indicated by arrows. (B) [^{32}P]-labeled poly(P) and Mfnk activities at various growth phases. The amount of [^{32}P]-labeled poly(P) was estimated as described in MATERIALS AND METHODS. Symbols: ○, *A*600 of culture; ●, log. of viable cell number; ■, specific activity of poly(P)-dependent NAD kinase activity, and □, amount of poly(P).

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Chapter II

Studies of ATP-NAD Kinase of *Escherichia coli*

Section 1 Purification of NAD kinase from *Escherichia coli* and identification as ATP-NAD kinase

In chapter I, a novel enzyme was isolated from *Micrococcus flavus* that utilizes inorganic polyphosphate [poly(P)] in addition to ATP for the phosphorylation of NAD:

$$\text{NAD} + \begin{pmatrix} \text{poly(P)n} \\ \text{ATP} \end{pmatrix} \rightarrow \text{NADP} + \begin{pmatrix} \text{poly(P)n-1} \\ \text{ADP} \end{pmatrix}$$

and the enzyme was designated as "poly(P)/ATP-NAD kinase" to discriminate it from NAD kinase (EC 2.7.1.23) [ATP-NAD kinase] that uses ATP, but not poly(P), for the phosphorylation of NAD (1):

$$\text{NAD} + \text{ATP} \rightarrow \text{NADP} + \text{ADP}$$

However, where ATP-NAD kinase is ? Indeed NAD kinase has been completely purified from pigeon liver (2), pigeon heart (3), *Saccharomyces cerevisiae* (4), and *Candida utilis* (5), but they may be poly(P)/ATP-NAD kinases since their abilities to utilize poly(P) were not checked in their purification procedures (2-5). Comparison of the properties and structure of ATP-NAD kinase with those of poly(P)/ATP-NAD kinase would give us valuable information as to physiological functions and catalytic mechanism, especially mechanism to utilize phosphoryl donors, of NAD kinase.

Therefore, attempts were made to isolate ATP-NAD kinase in microorganisms. *Escherichia coli* was chosen as a source of ATP-NAD kinase, since poly(P)-dependent NAD kinase activity was not detected in cell extract of this organism (6). In the study described in this Section, NAD kinase was isolated from the cell extract of *E. coli* through measuring ATP-dependent NAD kinase activity, shown to utilize ATP efficiently, but not poly(P), and identified as ATP-NAD kinase that was discriminated from poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* MG1655 was cultured at 37 ℃ in YGD medium (pH 7.2) comprising 0.10 % $(NH_4)_2SO_4$, 0.050 % $MgSO_4 7H_2O$, 0.10 % KH_2PO_4, 0.40 % Na_2HPO_4, 0.50 % yeast extract, and 0.50 % glucose.

Assays. NAD kinase activity (ATP-dependent NAD kinase activity) was assayed by means of a two-step method as described (6) in a reaction mixture (1.0 ml) consisting of 5.0 mM NAD, 5.0 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.0), and 5.0 mM ATP at 37 ℃, unless otherwise stated. NADP was enzymatically determined with isocitrate dehydrogenase (6). NADH kinase activity was assayed as described in Section 1 of Chapter I. One unit of enzyme activity was defined as 1.0 μmol of NADP produced in 1 min at 37 ℃, and specific activity was expressed in units/mg protein. K_m and V_{max} were determined by means of Lineweaver-Burk plots. k_{cat} was defined as $V_{max} \cdot [e_0]^{-1}$, where $[e_0]$ is the concentration of purified NAD kinase (M). Protein concentrations were determined by the method of Bradford (7) with bovine serum albumin as a standard.

Purification of NAD kinase from *E. coli*. Centrifugation was carried out at 20,000 x g, 4 ℃ for 20 min, and dialysis at 4 ℃ overnight against KND (10 mM potassium phosphate (pH 7.0), 0.10 mM NAD, and 0.50 mM dithiothreitol). Cells (36 g wet wt.) of *E. coli* MG1655 grown aerobically at 37 ℃ for 18 h in 14 l YGD medium were collected, suspended in 60 ml of KND, and then disrupted with Sonifire (Branson, Danbury, CT). The cell extract obtained on centrifugation was supplemented with 1.0 mM phenylmethylsulfonyl fluoride and then fractionated with ammonium sulfate (0 – 40 %). The precipitate was dissolved in KND, dialyzed, and then applied onto a DEAE-Toyopearl 650M column (2.7 x 18 cm) (Tosoh, Tokyo, Japan) equilibrated with KND. The non-absorbed fraction containing ATP-dependent NAD kinase activity was loaded onto an AF-Blue Toyopearl 650 ML column (0.8 x 20 cm) (Tosoh) equilibrated with KNDMg (KND containing 10 mM $MgCl_2$), and the enzyme was first eluted with a linear gradient of NaCl in KNDMg (0 – 3.0 M, 40 ml) and then with 80 ml of KNDMg containing 3.0 M NaCl in 2.0 ml portions every 2.0 min. The fractions containing the activities (fraction nos. 18 - 57) were combined, concentrated to about 6.0 ml by ultrafiltration with an Amicon model 8200 (Amicon Division, Beverly, MA), and then loaded onto a Sephacryl S-200 HR column (2.7 x 54 cm) (Amersham Pharmacia Biotech, Buckinghamshire, England) equilibrated with KND containing 150 mM NaCl. The

enzyme was eluted with the same buffer in 3.0 ml fractions every 4.4 min, and the fractions containing the activities (fraction nos. 57 - 62) were combined and then dialyzed against KND. The dialysate was applied onto a DEAE-Toyopearl 650M column (0.80 x 5.0 cm) equilibrated with KND and the enzyme was eluted with a linear gradient of NaCl (0 - 400 mM, 16 ml) in KND in 1.0 ml fractions every 1.0 min. The fractions containing the activities, which were obtained by elution with 130 - 160 mM NaCl, were combined, dialyzed, and used as a purified enzyme.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (8). Proteins in the gel were visualized with Coomassie Brilliant Blue R-250. The molecular mass of *E. coli* NAD kinase was estimated by gel filtration chromatography on a Sephacryl S-200 HR column (2.7 x 54 cm). The purified protein was loaded on to the column and eluted with 10 mM potassium phosphate (pH 7.0) containing 150 mM NaCl in 3.0 ml fractions every 6.0 min. Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) was used as standard proteins. N-terminal amino acid sequence was analyzed with a Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA). Blast (9) on the World-Wide Web site of the *E. coli* WWW Home Page (http://dna.aist-nara.ac.jp/ecoli/orfsearch.asp) was used for the homology search. Sequence data were obtained from the GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) and SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today) databases.

RESULTS

Purification of NAD kinase from *E. coli*. Cell extract of *E. coli* MG1655 showed ATP-dependent NAD kinase activity, but no poly(P)-dependent one. Thus, by measuring ATP-dependent NAD kinase activity, NAD kinase was purified approximately 3,400 fold from the cell extract of this organism (Table 1). The purified enzyme migrated as a single protein band corresponding to 30 kDa on SDS-PAGE (Fig. 1), and was eluted as a single peak of a 180 kDa protein on gel filtration chromatography (data not shown), indicating that the enzyme was a hexamer consisting of 30 kDa subunits.

TABLE 1 Purification of NAD kinase from *E. coli* MG1655

Step	Total protein (mg)	Total activity (units)	Activity yield (%)	Specific activity (units/mg)	Purification (fold)
Cell extract	2,385	1.06	100	0.00044	1.0
Ammonium sulfate	875	0.56	52.0	0.00064	1.5
DEAE-Toyopearl	239	0.46	43.4	0.00192	4.4
AF-Blue Toyopearl	8.80	0.63	58.9	0.0716	163
Sephacryl S-200	0.56	0.37	34.5	0.661	1,502
DEAE-Toyopearl	0.08	0.12	11.3	0.150	3,409

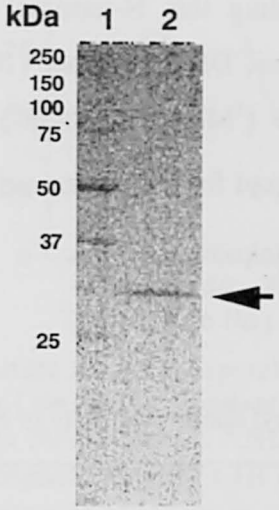

FIG. 1 SDS-PAGE of *E. coli* NAD kinase. Lane 1: Protein markers (Bio-Rad Laboratories, Hercules, CA). Lane 2: Purified enzyme (3.0 μg). Arrow indicates *E. coli* NAD kinase.

Characterization of ATP-NAD kinase of *E. coli*. NAD kinase of *E. coli* utilized ATP and other nucleoside triphosphates, but poly(P) with much lower efficiency (Table 2). The NAD kinase was therefore designated as "ATP-NAD kinase". Nucleoside diphosphates, glucose-6-phosphate, *p*-nitrophenylphosphate, and other energy-rich compounds (phosphocreatine and phosphoenolpyruvate) were also inert as phosphoryl donors (Table 2). Kinetic constants of the enzyme was as follows, K_m and k_{cat} were 2.50 mM, 55.0 s^{-1} for ATP, and 2.00 mM, 125 s^{-1} for NAD, respectively. Optimum pH and temperature were 7.5 in Tris-HCl (Fig. 2A) and 60 ℃ (Fig. 2B), respectively. Half of the activity was lost on treatment at 65 ℃ for 10 min (Fig. 2C). Bivalent metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} were indispensably required for the activity (Table 3). *p*-Chloromercuribenzoate and HgCl2 inhibited the activity (Table 4), indicating that the SH group of the enzyme plays

important roles. Dithiothreitol, 2-mercaptetanol, and reduced glutathione gave no effect on the activity at 1.0 mM. Although the inhibition of the enzyme by NADP was only slight, NADH and NADPH inhibited the enzyme at low concentrations (Table 4). Thus effects of NADH and NADPH on the activity of the enzyme were investigated. In the presence of NADPH or NADH, NAD saturation curve of the enzyme was transformed from a hyperbolic to a sigmoidal shape (Fig. 3A). Hill coefficients (h) were calculated to be 1.3 (in the presence of 5.0 μM NADH), and 1.5 (in the presence of 5.0 μM NADPH) (Fig. 3B). Phosphorylation of NADH to NADPH by the enzyme was not detected with TLC and HPLC analyses (data not shown).

N-terminal amino acid sequence of the ATP-NAD kinase was determined to be ^{1}MDNHFKCIGI^{10}. A gene encoding this N-terminal sequence was searched for in the database of the *E. coli* whole genomic DNA sequence (10), and this N-terminal sequence was found to be identical with that (^{1}MNNHFKCIGI^{10}) of YfjB (GenBank: D90888-18, AE000347; SwissProt P37768), except for one amino acid residue (^{2}N→^{2}D). YfjB had been deposited in databases as function-unknown protein.

TABLE 2 Phosphoryl donor specificity of *E. coli* ATP-NAD kinase

Phosphoryl donor	Relative activity (%)
UTP	109
ATP	100
CTP	60
GTP	56
dATP	42
TTP	40
ADP	nd
AMP	nd
Phosphocreatine	nd
Phosphoenolpyruvate	nd
p-Nitrophenylphosphate	nd
Glucose-6-phosphate	nd
Metaphosphate	2.4

NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of the phosphoryl donors listed above. Metaphosphate was obtained from Wako Pure Chemical Industries (Osaka, Japan), and was used at 1.0 mg/ml as poly(P). Other phosphoryl donors were purchased from Sigma (St. Louis, MO) and used at 5.0 mM. Activity for 5.0 mM ATP was taken as 100 %. nd, not detected.

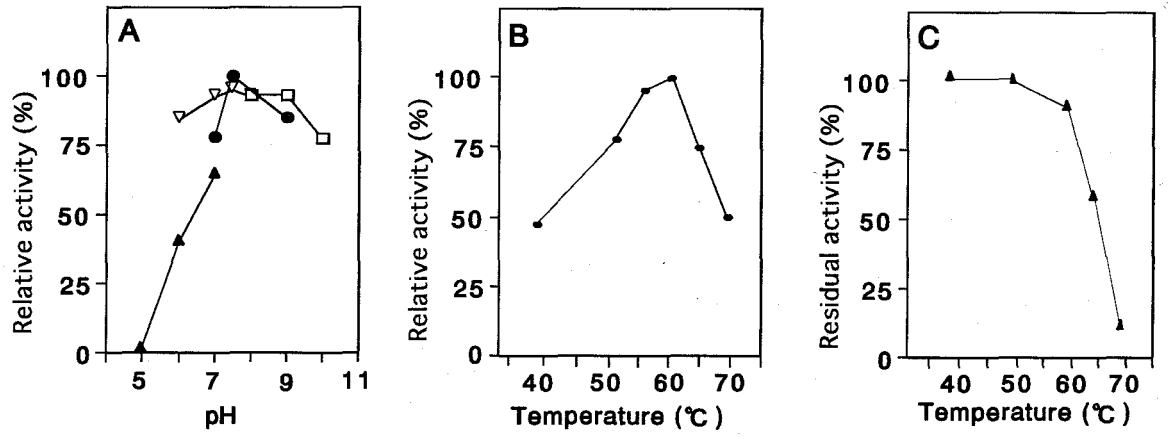

FIG. 2 Effects of pH and temperature on ATP-dependent NAD kinase activity of *E. coli* ATP-NAD kinase. (A) Effect of pH on ATP-dependent NAD kinase activity. NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of 100 mM sodium acetate (▲), potassium phosphate (▽), Tris-HCl (●), and glycine-NaOH (□). (B) Effect of temperature on ATP-dependent NAD kinase activity. NAD kinase activity was assayed as described in MATERIALS AND METHODS at each of the indicated temperatures. (C) Thermal stabilitiy of *E. coli* ATP-NAD kinase. The purified enzyme was incubated for 10 min at each of the indicated temperatures and then the residual activity was assayed as described in MATERIALS AND METHODS.

TABLE 3 Effects of metal ions on ATP-dependent activity of *E. coli* ATP-NAD kinase

Metal	Relative activity (%)
None	0
$MgCl_2$	100
$MnCl_2$	242
$ZnCl_2$	104
$CaCl_2$	85
$CoCl_2$	73
$FeCl_2$	nd
$CuCl_2$	nd
NaCl	nd
KCl	nd
LiCl	nd

ATP-dependent NAD kinase activity was assayed in the reaction mixture described in MATERIALS AND METHODS, in which 5.0 mM $MgCl_2$ was replaced for each of 1.0 mM metal ions listed above. Activity in the presence of 1.0 mM $MgCl_2$ was relatively taken as 100 %. nd, not detected.

TABLE 4 Effects of various compounds on the ATP-dependent NAD kinase activity of *E. coli* ATP-NAD kinase

Compound	Conc. (mM)	Relative activity (%)
None	0	100
NADPH	0.005	58
	0.010	24
NADH	0.005	68
	0.010	39
NADP	0.050	83
	0.100	79
p-Chloromercuribenzoate	1.0	nd
$HgCl_2$	1.0	nd

ATP-dependent NAD kinase activity was assayed in the reaction mixture described in MATERIALS AND METHODS containing each of various compounds listed above at the indicated concentrations. When each of *p*-chloromercuribenzoate and $HgCl_2$, inhibitors for isocitrate dehydrogenase, was present in the reaction mixture, NADP was enzymatically determined with glucose-6-phosphate dehydrogenase. Activity in the absence of the compounds was taken as 100 %. nd, not detected.

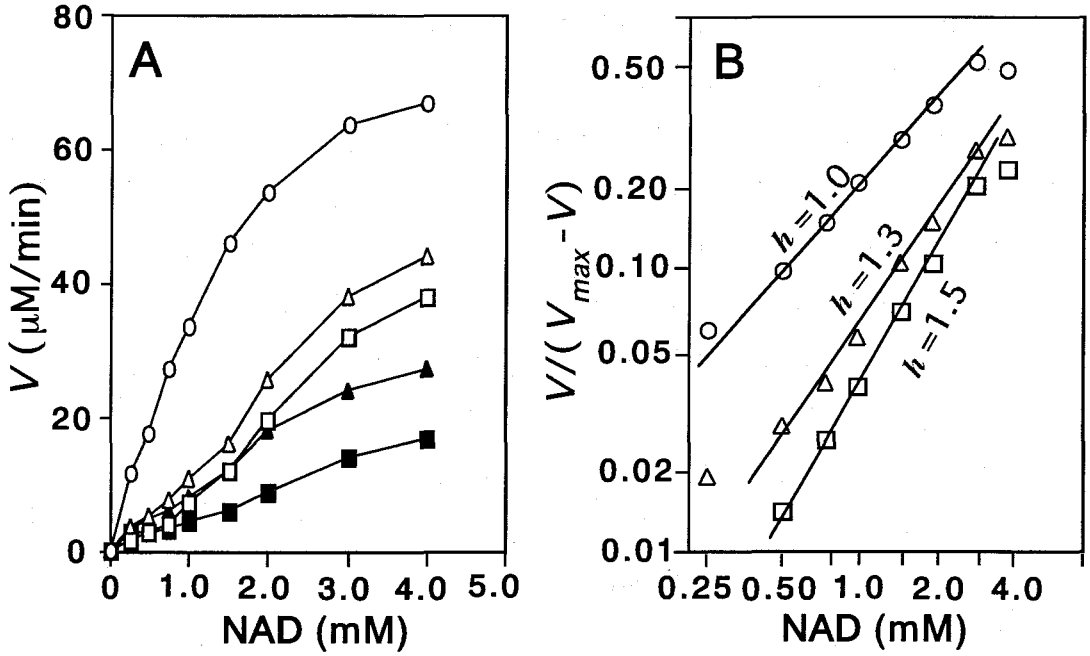

FIG. 3 Effects of NADPH and NADH on ATP-dependent NAD kinase activity of *E. coli* ATP-NAD kinase. (A) Effects of NADPH and NADH on NAD saturation curve of *E. coli* ATP-NAD kinase. (B) Hill plots of the data in panel A. NAD kinase activity was assayed in the reaction mixture described in MATERIALS AND METHODS in the presence of NADPH [0 μM (○), 5.0 μM (□), and 10 μM (■)] or NADH [5.0 μM (△) and 10 μM (▲)]. Hill coefficients (h) in the presence of 10 μM NADH or NADPH could not be definitely determined due to the strong inhibitory effects of NADH or NADPH.

DISCUSSION

NAD kinase was purified to homogeneity from *E. coli* MG1655, and shown to utilize ATP, but poly(P) with much lower efficiency. The enzyme was therefore identified as ATP-NAD kinase that is distinguishable from poly(P)/ATP-NAD kinase of *M. flavus* (Mfnk) described in Chapter I. In *E. coli* poly(P) is supposed to function as a regulator for stress and survival, and not as an ATP substitute (11). Inability of *E. coli* ATP-NAD kinase to utilize poly(P) is in accordance with the proposed function of poly(P) in *E. coli*.

Isolation of ATP-NAD kinase indicates that at least two kinds of NAD kinase, poly(P)/ATP-NAD kinase and ATP-NAD kinase, exist in living organisms. Like Mfnk (Chapter I), *E. coli* ATP-NAD kinase did not utilize glucose-6-phosphate and *p*-nitrophenylphosphate as phosphoryl donors (Table 2), although NAD-phosphorylating activities which utilize *p*-nitrophenylphosphate and glucose-6-phosphate were detected in cell extracts of *Proteus mirabilis* (12) and coryneform bacteria isolated from sewage sludge (13), respectively.

Cellular contents of NAD, NADP, NADH, and NADPH in *E. coli* at exponential growth phase have been estimated to be 0.90, 0.17, 0.40, and 0.22 mM, respectively (14). The K_m of *E. coli* ATP-NAD kinase for NAD was calculated to be 2.00 mM (Table 5). Although being comparable with that of partially purified *E. coli* NAD kinase (1.90 mM) (15), the K_m was higher than the physiological concentration of NAD (0.90 mM) (15) in *E. coli*. Furthermore, NADPH and NADH strongly inhibited the activity of *E. coli* ATP-NAD kinase (Table 4) and shifted a NAD saturation curve of the enzyme to a sigmoidal shape (Fig. 3A) at less than their physiological concentrations in *E. coli*. These behaviors of the enzyme, which were also observed in the case of the partially purified *E. coli* NAD kinase (15), indicate that *E. coli* ATP-NAD kinase is an allosteric enzyme and that NADPH and NADH are the effective allosteric modifiers for the enzyme. The facts described above, i.e. higher K_m, strict inhibitions by NADPH and NADH, and allosteric behavior of *E. coli* ATP-NAD kinase, would indicate that the physiological activity of the enzyme is strictly repressed and regulated in *E. coli* cell, presumably to keep both [NADPH]/[NADP] and [NAD]/[NADH] at high levels. As described in previous Chapter, poly(P)/ATP-NAD kinase of *M. flavus* could utilize NADH as phosphoryl acceptor, while *E. coli* ATP-NAD kinase not. This fact indicates that in *E. coli* syntheses of NADP and NADPH are regulated by distinct manner from that of *M. flavus*.

N-terminal amino acid sequence of *E. coli* ATP-NAD kinase was found to be identical with that of YfjB, which had been deposited as a function-unknown protein in the database of the *E. coli* whole genomic DNA sequence, suggesting that YfjB is ATP-NAD kinase. Whether or not YfjB is ATP-NAD kinase is described in the next Section (Section 2 in Chapter II)

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Section 2 Molecular cloning of ATP-NAD kinase gene of *Escherichia coli*

In Section 1 of this Chapter, NAD kinase was isolated from *Escherichia coli* MG1655 and identified as ATP-NAD kinase. N-terminal amino acid sequence of the enzyme was determined to be ^{1}MDNHFKCIGI^{10}. As a result of a homology search in a database of the *E. coli* whole genomic DNA sequence (1), this N-terminal sequence was found identical with that (^{1}MNNHFKCIGI^{10}) of YfjB (GenBank: D90888-18, AE000347; SwissProt: P37768) except for one amino acid residue (^{2}N→^{2}D), suggesting YfjB is ATP-NAD kinase. YfjB has been deposited as a function-unknown protein and coded by a gene, *yfjB*. In the study described in this Section, *yfj*B was cloned and identified as ATP-NAD kinase gene.

MATERIALS AND METHODS

Bacterial strains. *E. coli* MG1655 and *E. coli* DH5α (Toyobo, Osaka, Japan) were cultured at 37 ℃ in Luria-Bertani (LB) medium (2). For the cultivation of *E. coli* DH5α, the medium was supplemented with ampicillin (100 μg/ml). The growth conditions for the derivative strains of *E. coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany) are described in the text.

Assays. NAD kinase activity was assayed as described (3) and protein concentrations were as described by Bradford (4). Details are described in Section 1 of this Chapter.

Cloning of *yfjB*. Genomic DNA of *E. coli* MG1655 was isolated as described (2). *yfjB* was amplified by PCR (Takara Biomedicals, Kyoto, Japan) from genomic DNA of *E. coli* in a reaction mixture (100 μl) comprising 2.5 U KOD polymerase (Toyobo), 0.25 μg genomic DNA, 40 pmol *yfjB Nco*I primer 5' AACCATGGATAATCATTTCAAGTGTATTGGCATTG 3', 40 pmol *yfjB Bam*HI primer 5' GCGGATCCTTAGAATAATTTTTTGACCAGCCGAG 3', 20 nmol dNTPs, 100 nmol MgCl2, 8.0 % dimethyl sulfoxide, and reaction buffer #1 supplemented with KOD polymerase. The cycle condition for PCR was as follows: 94 ℃ 30 sec, 50 ℃ 30 sec, and 74 ℃ 30 sec, 30 cycles. The PCR product (0.88 kb fragment) was separated by 0.80 % agarose gel electrophoresis, isolated with a gene clean kit (Bio 101, Vista, CA), and confirmed as *yfjB* after nucleotide sequence. The PCR product was digested with *Nco*I and *Bam*HI, and then ligated into pET-14b (Novagen) digested with *Nco*I and *Bam*HI,

which yielded pSK67. Derivative strains, SK67 and SK45, were obtained by transforming *E. coli* BL21(DE3)pLysS with pSK67 and pET-14b, respectively.

Expression of YfjB in *E. coli*. For the expression of YfjB, SK67 was inoculated into 480 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chroramphenicol, and then cultured at 37 ℃ aerobically until A_{600} reached 1.0. The culture was then transferred to 13.5 l of the same medium with the same antibiotics and cultivation was continued at 37 ℃ aerobically for 2 h until A_{600} reached 0.70; then isopropyl-β-D-thiogalactopyranoside was added to give a final concentration of 0.10 mM, and the cultivation was continued further at 16 ℃ aerobically for 36 h. As a control, SK45 in a 10 ml culture was also treated with the same manner.

Purification of YfjB expressed in *E. coli*. Centrifugation was carried out at 20,000 x g, 4 ℃ for 20 min, and dialysis at 4 ℃ overnight against KND. YfjB expressed in *E. coli* was purified by measuring ATP-dependent NAD kinase activity as follows. Cells of SK67 (119 g wet wt.) expressing YfjB were collected, suspended in 100 ml KND, and disrupted by Sonifire (Branson, Danbury, CT) as above. The cell extract of SK67 was, after centrifugation, supplemented with 1.0 mM phenylmethylsulfonyl fluoride and then fractionated with ammonium sulfate (0 – 40 %). The precipitate was dissolved in KND, dialyzed, and then applied onto a DEAE-Toyopearl 650M column (4.2 x 17.5 cm) (Tosoh, Tokyo, Japan) equilibrated with KND. YfjB was eluted with a linear gradient of NaCl (0 - 500 mM, 800 ml) in KND in 8.0 ml fractions every 4.0 min. The fractions containing YfjB, which were obtained by elution with 160 - 250 mM NaCl, were combined, saturated with ammonium sulfate (25 %), and then directly applied onto a Butyl-Toyopearl 650M column (2.6 x 13.5 cm) (Tosoh) equilibrated with KND containing 25 % ammonium sulfate. YfjB was eluted with a linear gradient of ammonium sulfate (25 – 0 %, 320 ml) in KND in 4.0 ml fractions every 4.0 min. The fractions containing YfjB, which were obtained by elution with 10 – 4.0 % ammonium sulfate, were combined, concentrated by ultrafiltration with an Amicon model 8200 (Amicon, Beverly, MA), dialyzed, and used as a purified YfjB.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (5). Proteins in the gel were visualized with Coomassie Brilliant Blue R-250. The molecular mass of YfjB was estimated by gel filtration chromatography on a Sephacryl S-200 HR column (2.7 x 54 cm) (Amersham Pharmacia Biotech, Buckinghamshire, England). The purified protein was loaded on to the column and eluted with 10 mM potassium phosphate (pH 7.0) containing 150

mM NaCl in 3.0 ml fractions every 6.0 min. Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) was used as standard proteins. N-terminal amino acid sequence was analyzed with a Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA). DNA sequence was determined with an automated DNA sequencer (Model 377; Applied Biosystems Division of Perkin-Elmer). Sequence data were obtained from the GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) and SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today) databases.

RESULTS AND DISCUSSION

Identification of *yfjB* as ATP-NAD kinase gene. YfjB is a polypeptide of 292 amino acids with a calculated molecular mass of 32,566 Da. *yfjB*, which had been deposited in databases as a gene of unknown function (GenBank: D90888-18, AE000347, SwissProt: P37768), has a start codon that is preceded by a probable ribosome binding site (AAGGA), and a stop codon followed a hairpin loop structure (Fig. 1). In order to confirm that *yfjB* codes for ATP-NAD kinase, *yfjB* was cloned from genomic DNA of *E. coli* MG1655 and expressed in *E. coli* BL21(DE3)pLysS as described in MATERIALS AND METHODS. A cell extract of SK67, which was expected to express *yfjB*, showed exceedingly high NAD kinase activity (4.82 units/mg). On the other hand, that of a control strain, SK45, exhibited an extremely low level of NAD kinase activity (0.0042 units/mg). In accordance with these results, when cell extracts of SK67 and SK45 were analyzed by SDS-PAGE, a protein of 30 kDa was observed in the cell extract of SK67 (Fig. 2B, lane 3), but not in that of SK45 (Fig. 2B, lane 2). It was therefore concluded that *yfjB* was expressed in *E. coli* BL21(DE3)pLysS and the expressed product (YfjB) was an NAD kinase with a subunit molecular mass of 30 kDa.

YfjB was purified to homogeneity (60 fold) from a cell extract of SK67 as described in MATERIALS AND METHODS (Table 1). YfjB comprised 30 kDa subunits (Fig. 2B, lane 4) and, on gel filtration chromatography, was eluted as a single peak corresponding to 180 kDa (data not shown). In addition, N-terminal amino acid sequence and other properties of YfjB were almost the same as those of ATP-NAD kinase purified from *E. coli* MG1655, indicating that *yfjB* surely encodes ATP-NAD kinase. This was the first cloning of ATP-

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        10         20         30         40         50         60         70         80
atgaataatcatttcaagtgtattggcattgtgggacacccacggcaccccactgcactgacaacacatgaaatgctcta
M  N  N  H  F  K  C  I  G  I  V  G  H  P  R  H  P  T  A  L  T  T  H  E  M  L  Y
        90        100        110        120        130        140        150        160
ccgctggctgtgcacaaaaggttacgaggtcatcgttgagcaacaaatcgctcacgaactgcaactgaagaatgtgaaaa
 R  W  L  C  T  K  G  Y  E  V  I  V  E  Q  Q  I  A  H  E  L  Q  L  K  N  V  K  T
       170        180        190        200        210        220        230        240
ctggcacgctcgcggagattgggcaactagctgatctcgcggtagtcgttggtggcgacggtaatatgctgggcgcggca
  G  T  L  A  E  I  G  Q  L  A  D  L  A  V  V  V  G  G  D  G  N  M  L  G  A  A
       250        260        270        280        290        300        310        320
cgcacactcgcccgttacgatattaaagttattggaatcaaccgtggcaacctgggtttcctgactgaccttgaccccga
R  T  L  A  R  Y  D  I  K  V  I  G  I  N  R  G  N  L  G  F  L  T  D  L  D  P  D
       330        340        350        360        370        380        390        400
taacgcccagcaacagttagccgatgtgctggaaggccactacatcagcgagaaacgttttttgctggaagcgcaagtct
 N  A  Q  Q  Q  L  A  D  V  L  E  G  H  Y  I  S  E  K  R  F  L  L  E  A  Q  V  C
       410        420        430        440        450        460        470        480
gtcagcaagattgccagaaacgcatcagcaccgcgataaatgaagtggtgcttcatccaggcaaagtggcgcatatgatt
  Q  Q  D  C  Q  K  R  I  S  T  A  I  N  E  V  V  L  H  P  G  K  V  A  H  M  I
       490        500        510        520        530        540        550        560
gagttcgaagtgtatatcgacgagatctttgcgttttctcagcgatctgatggactaattatttcgacgccaacaggctc
E  F  E  V  Y  I  D  E  I  F  A  F  S  Q  R  S  D  G  L  I  I  S  T  P  T  G  S
       570        580        590        600        610        620        630        640
caccgcctattccctctctgcaggcggtcctattctgacccctctctggatgcgattaccctggtgcccatgttcccgc
 T  A  Y  S  L  S  A  G  G  P  I  L  T  P  S  L  D  A  I  T  L  V  P  M  F  P  H
       650        660        670        680        690        700        710        720
atacgttgtcagcacgaccactggtcataaacagcagcagcacgatccgtctgcgtttttcgcatcgccgtaacgacctg
  T  L  S  A  R  P  L  V  I  N  S  S  S  T  I  R  L  R  F  S  H  R  R  N  D  L
       730        740        750        760        770        780        790        800
gaaatcagttgcgacagccagatagcactgccgattcaggaaggtgaagatgtcctgattcgtcgctgtgattaccatct
E  I  S  C  D  S  Q  I  A  L  P  I  Q  E  G  E  D  V  L  I  R  R  C  D  Y  H  L
       810        820        830        840        850        860        870
gaatctgattcatccgaaagattacagttatttcaacacattaagcaccaagctcggctggtcaaaaaaattattctaa
 N  L  I  H  P  K  D  Y  S  Y  F  N  T  L  S  T  K  L  G  W  S  K  K  L  F  *
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FIG. 1 Nucleotide sequence of *yfjB*. Deduced amino acid sequence of YfjB is indicated below nucleotide sequence.

NAD kinase. *E. coli yfjB* mutant was failed to obtain (data not shown), which would suggest that *yfjB* is essential for the proliferation of *E. coli* cells. Features of primary structure of YfjB are discussed in Section 3 of Chapter III.

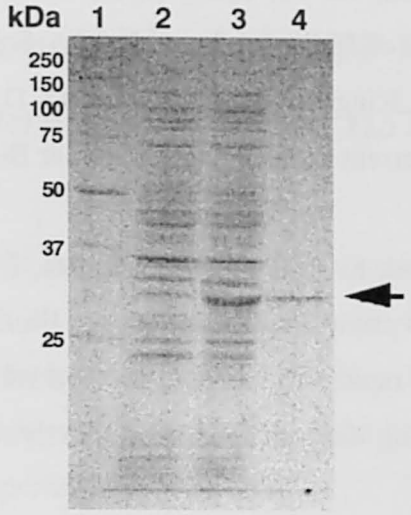

FIG. 2 SDS-PAGE of *E. coli* ATP-NAD kinase (YfjB) purified from SK67. Lane 1: Protein markers (Bio-Rad Laboratories, Hercules, CA). Lane 2: Cell extract of SK45 (control strain, 15.0 μg). Lane 3: Cell extract of SK67 (15.0 μg). Lane 4: Purified YfjB (3.0 μg). YfjB is indicated by an arrow.

TABLE 1 Purification of YfjB from SK67

Step	Total protein (mg)	Total activity (unit)	Yield (%)	Specific activity (units/mg)	Purification (fold)
Cell extract	3,135	15,100	100	4.82	1.0
Ammonium sulfate	1,297	9,166	57.3	7.07	1.5
DEAE-Toyopearl	450	5,918	39.0	13.2	2.7
Butyl-Toyopearl	60	789	5.2	13.2	2.7

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Chapter III

Molecular Structures of Microbial NAD Kinases

Section 1 Inorganic polyphosphate/ATP-NAD kinase of *Mycobacterium tuberculosis* H37Rv

In the studies described in the previous Chapters, inorganic polyphosphate [poly(P)]/ATP-NAD kinase and ATP-NAD kinase were purified and their genes were cloned from *Micrococcus flavus* and *Escherichia coli*, respectively. Comparison of structures and properties of these NAD kinases with those of other NAD kinases would further deepen our insight as to structures and properties of NAD kinase.

N-terminal and internal amino acid sequences of poly(P)/ATP-NAD kinase (Mfnk) purified from *M. flavus* showed homologies with many amino acid sequences of function-unknown proteins including Rv1695 protein (1) (GenBank: Z98268-16, SwissProt: O33196) of *Mycobacterium tuberculosis* H37Rv and Utr1p (GenBank: L26347-2, Z49549-1; SwissProt: P21373) of *Saccharomyces cerevisiae.*

Rv1695 protein, which was encoded by a gene named Rv1695, was proposed as a candidate for poly(P)-dependent NAD kinase on the two bases: (i) *M. tuberculosis* H37Rv possessed poly(P)/ATP-glucokinase (2); (ii) microorganisms exhibiting poly(P)-dependent glucokinase activities tend to possess enzymes responsible for poly(P)-dependent NAD kinase ones (3, 4). In the study described in this section, Rv1695 was cloned and identified as poly(P)/ATP-NAD kinase gene. Properties of Rv1695 protein [poly(P)/ATP-NAD kinase of *M. tuberculosis*] purified from *E. coli* were compared with those of Mfnk of *M. flavus.*

MATERIALS AND METHODS

Strains. Cultivation conditions of the derivative strains of *E. coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany) are described in the text. As a host for plasmid amplification, *E. coli* DH5α (Toyobo, Osaka, Japan) was routinely cultured at 37 ℃ in Luria-Bertani (LB) medium (5) with ampicillin (100 μg/ml).

Assays. Poly(P)- and ATP-dependent NAD kinase activities were assayed as described

(3) and Section 1 of Chapter I. NADH kinase activity was assayed as described in Section 1 of Chapter I. Protein concentration was determined by the method of Bradford (6) with bovine serum albumin as a standard. Phosphatase activity of the enzyme was assayed by measuring orthophosphate released as described (7) in an above-described reaction mixture without NAD.

Amino acid sequence analysis. For N-terminal amino acid sequence, the purified enzyme was directly analyzed with Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA).

DNA sequence analysis. DNA sequence was determined using an automated DNA sequencer (Model 377; Applied Biosystems Division of Perkin-Elmer).

Cloning of Rv1695. Genomic DNA of *M. tuberculosis* H37Rv was kindly given by Dr. Takeshi Yamada. Rv1695 was amplified from the DNA with PCR (Takara Biomedicals, Kyoto, Japan). PCR was performed in a reaction mixture (100 μl) containing 2.5 U KOD polymerase (Toyobo, Osaka, Japan), 0.25 μg *M. tuberculosis* H37Rv genomic DNA, 40 pmol *Nde*I primer 5' CCCATATGACCGCTCATCGCAGTGTTCTG 3', 40 pmol *Bam*HI primer 5' CGGATCCCTACTTTCCGCGCCAACCGGTC 3', 20 nmol dNTPs, 100 nmol MgCl2, 8.0 % dimethyl sulfoxide, and the reaction buffer #1 supplied with KOD polymerase. Cycle condition of PCR was as follows: 98 ℃ 15 sec, 67 ℃ 2 sec, 74 ℃ 30 sec, 25 cycles. PCR products were separated on a 0.80 % agarose gel electrophoresis and 0.93 kb PCR product was isolated with gene clean kit (Bio 101, Vista, CA). The nucleotide sequence of 0.93 kb PCR product was determined and identified to be Rv1695. The 0.93 kb PCR product was digested with *Nde*I and *Bam*HI and ligated into pET-3a (Novagen) digested with *Nde*I and *Bam*HI, yielding pSK27. Derivative strains, SK27 and SK35 (control), were obtained by transforming *E. coli* BL21(DE3)pLysS with pSK27 and pET-3a, respectively.

Expression of Rv1695 protein in *E. coli*. For the expression of Rv1695 protein, SK27 was inoculated into 400 ml LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chroramphenicol, and cultured at 37 ℃ aerobically until A_{600} reached 1.4. This culture was transferred into 13.5 l of the same medium with the same antibiotics and cultivation was continued at 37 ℃ aerobically for 2 h until the A_{600} reached 0.70; then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.40 mM, and the cultivation was continued again at 18 ℃ aerobically for 3 days. As a control, SK35 in a 10 ml medium was also treated with the same manner.

Purification of Rv1695 protein expressed in *E. coli*. Centrifugation was carried out at

20,000 x g, 4 ℃, for 20 min and dialysis was at 4 ℃ overnight against KNDE (10 mM potassium phosphate (pH 7.0), 0.10 mM NAD, 0.50 mM dithiothreitol, and 1.0 mM EDTA). Rv1695 protein was purified by measuring poly(P)- and ATP-dependent NAD kinase activities. Cell extract of SK27 expressing Rv1695 protein was fractionated with ammonium sulfate (20 – 40 %). The precipitate was dissolved in 50 ml of KNDE, dialyzed, and then the solution was applied onto a DEAE-Toyopearl 650M column (5.0 x 27 cm) (Tosoh, Tokyo, Japan) equilibrated with KNDE. Rv1695 protein was eluted with a linear gradient of NaCl in KND (0–700 mM, 1,600ml). The fractions with Rv1695 protein, which were obtained by elution with 350 – 450 mM NaCl, were combined, saturated with ammonium sulfate (30 %), and then directly applied onto a Butyl-Toyopearl 650M column (2.7 x 10 cm) (Tosoh). Rv1695 protein was eluted with a linear gradient of ammonium sulfate in KND (30 – 0 %, 150 ml). The fractions with Rv1695 protein, which were obtained by elution with 20 - 15 % ammonium sulfate, were combined, dialyzed, and used as a purified poly(P)/ATP-NAD kinase (Ppnk) of *M. tuberculosis* H37Rv.

Other analytical methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (8). Proteins in the gel were visualized by Coomassie Brilliant Blue R-250. Molecular mass of the enzyme was estimated by gel filtration chromatography on Sephacryl S-200 HR column (2.7 x 54 cm) (Amersham Pharmacia Biotech, Buckinghamshire, England) with Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) as recommended by manufacturer. Fasta (9) and Blast (10) on World-Wide Web site of the DNA Databank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html) were used for homology search. Sequences were obtained from GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) or SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today). Molecular mass of polypeptide was calculated with GENETYX program (Software Development, Tokyo, Japan).

RESULTS AND DISCUSSION

Identification of Rv1695 of *M. tuberculosis* H37Rv as poly(P)/ATP-NAD kinase gene (*ppnk*). The Rv1695 protein is a polypeptide of 307 amino acids with a calculated molecular mass of 32,873 Da and is encoded on a gene named Rv1695. Rv1695 consists of 924 nucleotides (Fig. 1), has start codon which is preceded by probable ribosome binding site (GAGG), and stop codon followed hairpin loop structure. To confirm the proposition that Rv1695 encodes poly(P)-dependent NAD kinase, Rv1695 was cloned from *M. tuberculosis* H37Rv genomic DNA with PCR and expressed in *E. coli* as described in MATERIALS AND METHODS. Cell extract of SK27 expressing Rv1695 protein showed 0.52 and 0.70 units/mg of poly(P)- and ATP-dependent NAD kinase activities, respectively, while that of control strain, SK35 exhibited 0.0042 units/mg ATP-dependent NAD kinase activity and no detectable poly(P)-dependent one, suggesting that poly(P)/ATP-NAD kinase is expressed. In cell extract of SK27, a major band of 35 kDa was found through SDS-PAGE analysis (Fig. 2, lane 2), while no such bands were observed in cell extract of control strain, SK35 (Fig. 2, lane 1). The Rv1695 protein (Fig. 2, lane 3), which was purified by measuring both poly(P)- and ATP-dependent NAD kinase activities from cell extract of SK27 (Table 1), also showed a single band of 35 kDa on SDS-PAGE (Fig. 2, lane 3). N-terminal amino acid sequence of the purified RV1695 protein with 35 kDa subunit is ^{1}TAHRSVLLVV^{10} that is identical with that deduced from Rv1695. From the expression, purification, and N-terminal amino acid analyses described above, it was concluded that Rv1695, which was named *ppnk*, encodes poly(P)/ATP-NAD kinase (Ppnk) with 35 kDa subunit.

On the gel filtration chromatography on Sephacryl S-200 HR column, the purified Rv1695 protein (Ppnk) was eluted as a 158 kDa protein (data not shown), indicating that Ppnk forms a tetramer consisting of 35 kDa subunits.

Properties of poly(P)/ATP-NAD kinase (Ppnk). Poly(P)/ATP-NAD kinase (Ppnk) of *M. tuberculosis* H37Rv purified from SK27 was characterized and properties of the enzyme were compared with those of poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus* described in Chapter I. Ppnk completely phosphorylated NAD to NADP utilizing poly(P) or ATP (data not shown). ATP and dATP were utilized more effectively than other nucleoside triphosphates by Ppnk and Mfnk (Table 2). Commercially available poly(P)s were utilized by them, except for trimetaphosphate [cyclic form of tripolyphosphate] (Table 2).

```
        10        20        30        40        50        60        70        80
GTGACCGCTCATCGCAGTGTTCTGCTGGTCGTCCACACCGGGCGCGACGAAGCCACCGAGACCGCACGGCGCGTAGAAAA
V  T  A  H  R  S  V  L  L  V  V  H  T  G  R  D  E  A  T  E  T  A  R  R  V  E  K
        90       100       110       120       130       140       150       160
AGTATTGGGCGACAATAAAATTGCGCTTCGCGTGCTCTCGGCCGAAGCAGTCGACCGAGGGTCGTTGCATCTGGCTCCCG
 V  L  G  D  N  K  I  A  L  R  V  L  S  A  E  A  V  D  R  G  S  L  H  L  A  P  D
       170       180       190       200       210       220       230       240
ACGACATGCGGGCCATGGGCGTCGAGATCGAGGTGGTTGACGCGGACCAGCACGCAGCCGACGGCTGCGAACTGGTGCTG
  D  M  R  A  M  G  V  E  I  E  V  V  D  A  D  Q  H  A  A  D  G  C  E  L  V  L
       250       260       270       280       290       300       310       320
GTTTTGGGCGGCGATGGCACCTTTTTGCGGGCAGCCGAGCTGGCCCGCAACGCCAGCATTCCGGTGTTGGGCGTCAATCT
V  L  G  G  D  G  T  F  L  R  A  A  E  L  A  R  N  A  S  I  P  V  L  G  V  N  L
       330       340       350       360       370       380       390       400
GGGCCGCATCGGCTTTTTGGCCGAGGCCGAGGCGGAGGCAATCGACGCGGTGCTCGAGCATGTTGTCGCACAGGATTACC
 G  R  I  G  F  L  A  E  A  E  A  E  A  I  D  A  V  L  E  H  V  V  A  Q  D  Y  R
       410       420       430       440       450       460       470       480
GGGTGGAAGACCGCTTGACTCTGGATGTCGTGGTGCGCCAGGGCGGGCGCATCGTCAACCGGGGTTGGGCGCTCAACGAA
  V  E  D  R  L  T  L  D  V  V  V  R  Q  G  G  R  I  V  N  R  G  W  A  L  N  E
       490       500       510       520       530       540       550       560
GTCAGTCTGGAAAAGGGCCCGAGGCTCGGCGTGCTTGGGGTGGTCGTGGAAATTGACGGTCGGCCGGTGTCGGCGTTTGG
V  S  L  E  K  G  P  R  L  G  V  L  G  V  V  V  E  I  D  G  R  P  V  S  A  F  G
       570       580       590       600       610       620       630       640
CTGCGACGGGGTGTTGGTGTCCACGCCGACCGGATCAACCGCCTATGCATTCTCGGCGGGAGGCCCGGTGCTGTGGCCCG
 C  D  G  V  L  V  S  T  P  T  G  S  T  A  Y  A  F  S  A  G  G  P  V  L  W  P  D
       650       660       670       680       690       700       710       720
ACCTCGAAGCGATCCTGGTGGTCCCCAACAACGCTCACGCGCTGTTTGGCCGGCCGATGGTCACCAGCCCCGAAGCCACC
  L  E  A  I  L  V  V  P  N  N  A  H  A  L  F  G  R  P  M  V  T  S  P  E  A  T
       730       740       750       760       770       780       790       800
ATCGCCATCGAAATAGAGGCCGACGGGCATGACGCCTTGGTGTTCTGCGACGGTCGCCGCGAAATGCTGATACCGGCCGG
I  A  I  E  I  E  A  D  G  H  D  A  L  V  F  C  D  G  R  R  E  M  L  I  P  A  G
       810       820       830       840       850       860       870       880
CAGCAGACTCGAGGTCACCCGCTGTGTCACGTCCGTCAAATGGGCACGGCTGGACAGTGCGCCATTCACCGACCGGCTGG
 S  R  L  E  V  T  R  C  V  T  S  V  K  W  A  R  L  D  S  A  P  F  T  D  R  L  V
       890       900       910       920
TGCGCAAGTTCCGGTTGCCGGTGACCGGTTGGCGCGGAAAGTAG
  R  K  F  R  L  P  V  T  G  W  R  G  K  *
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FIG. 1 Nucleotide sequence of Rv1695. Deduced amino acid sequence of Rv1695 protein is indicated below nucleotide sequence.

TABLE 1 Purification of Rv1695 protein from SK27

Step	Total Protein (mg)	Poly(P)-dependent activity: Total activity (units)	Yield (%)	Specific activity (a) (units/mg)	Purification (fold)	ATP-dependent activity: Total activity (units)	Yield (%)	Specific activity (b) (units/mg)	Purification (fold)	Ratio (b/a)
Cell extract	8,100	4185	100	0.52	1.0	5670	100	0.70	1.0	1.4
AS[a] (20-40%)	2,300	2952	71	1.28	2.5	2223	39	0.97	1.4	0.8
DEAE-Toyopearl	1,260	1869	45	1.48	2.9	1785	32	1.42	2.0	1.0
Butyl-Toyopearl	100	308	7.4	3.08	6.0	302	5.3	3.02	4.3	1.0

[a] Ammonium sulfate

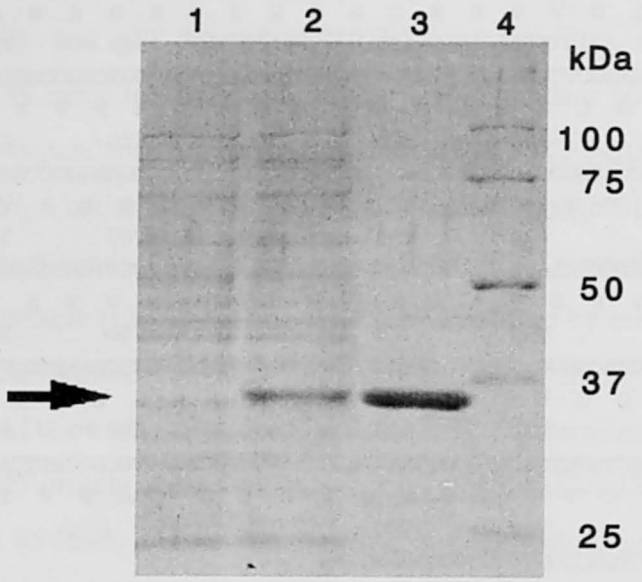

FIG. 2 SDS-PAGE of Rv1695 protein [poly(P)/ATP-NAD kinase (Ppnk)] of *M. tuberculosis* purified from SK27. Lane 1: Cell extract of SK35 (control strain, 5.0 μg). Lane 2: Cell extract of SK27 (5.0 μg). Lane 3: Purified Rv1695 protein (5.0 μg). Lane 4: Protein markers (Bio-Rad Laboratories, Hercules, CA). An arrow indicates Rv1695 protein [poly(P)/ATP-NAD kinase (Ppnk)] .

Furthermore, kinetic constants showed that Ppnk utilized ATP and poly(P) [tetrapolyphosphate, poly(P)4] with similar efficiencies, while Mfnk preferred ATP to poly(P) [poly(P)4] (Table 3). Kinetic constants of Ppnk were also compared with those of *E. coli* ATP-NAD kinase (YfjB) described in Chapter II (Table 4). k_{cat} (s^{-1}) of YfjB [55.0 (ATP)] was significantly higher than that of Ppnk [2.72 (ATP)], indicating that YfjB uses ATP more efficiently than Ppnk does. The ability of Ppnk to utilize poly(P) might compensate for the low ability of Ppnk to use ATP. *p*-Nitrophenylphosphate was not utilized by Ppnk and Mfnk (Table 2), indicating that NAD phosphorylation was not due to

TABLE 2 Phosphoryl donor specificities of Mfnk and Ppnk

Phosphoryl donor	Relative activity (%) Mfnk	Ppnk	Phosphoryl donor	Relative activity (%) Mfnk	Ppnk
ATP	100	100	Orthophosphate	nd	nd
dATP	91	96	Pyrophosphate	nd	nd
AMP	nd	nd	Trimetaphosphate	nd	nd
ADP	nd	nd	Tripolyphosphate	40	38
GTP	88	47	Poly(P)4	151	163
CTP	73	16	Phosphate glass (type 35)	49	13
dTTP	74	33	Polyphosphate	138	151
UTP	87	88	Hexametaphosphate	88	118
Glucose-6-phosphate	nd	nd	Metaphosphate	88	178
p-Nitrophenylphosphate	nd	nd			

NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of the phosphoryl donors listed above. Each of activity of Mfnk and Ppnk for 5.0 mM ATP was relatively taken as 100 %. Metaphosphate, hexametaphosphate and polyphosphate (Wako Pure Chemical Industries) were used at 1.0 mg/ml. Others were from Sigma (St. Louis, MO) and used at 5.0 mM. A number of phosphoryl residues and molecular weight of the phosphate glass (type 35) are estimated to be 32 and 3,292, respectively. nd, not detected.

the reverse (phosphatase) reactions of the enzymes (11). The enzymes did not show NAD kinase activities that utilize glucose-6-phosphate (Table 2), although such activities have been found in some coryneform bacteria isolated from sewage sludge (12). In the absence of NAD, the enzymes showed no phosphatase activities for ATP and poly(P)4. Both enzymes required bivalent metal ions such as Mg^{2+} and Mn^{2+} for their poly(P)- and ATP-dependent NAD kinase activities, and Mn^{2+} was more effective activator for both enzymes (Table 5). 1.0 mM *p*-chloromercuribenzoate completely inhibited poly(P)- and ATP-dependent NAD kinase activities of the enzymes. Optimum pH of Ppnk was 6.5 in sodium-acetate [for poly(P)-dependent NAD kinase activity], and 8.0 in Tris-HCl [for ATP-dependent NAD kinase activity], respectively (Fig. 3A). Optimum temperature of Ppnk was 50 ℃ irrespective of phosphoryl donors (Fig. 3B). Halves of both poly(P)- and ATP-dependent NAD kinase activities of Ppnk were lost on treatment at 50 ℃ for 10 min (Fig. 3C). Ppnk and Mfnk were inhibited by NADPH and NADH, while not affected by NADP (Table 6). Like Mfnk, Ppnk also showed both poly(P)- and ATP-dependent NADH kinase activities that were confirmed by TLC (data not shown) and HPLC (Fig. 4) analyses. ATP-dependent NADH kinase activity was 14. 5 % to ATP-dependent NAD kinase one, while poly(P)-

dependent NADH activity was 9.0 % to poly(P)-dependent NAD kinase one.

TABLE 3 Kinetic constants of Mfnk and Ppnk

Compound	K_m (mM)		V_{max} (μM/h)		Efficiency (V_{max}/K_m)		Relative efficiency (%)	
	Mfnk	Ppnk	Mfnk	Ppnk	Mfnk	Ppnk	Mfnk	Ppnk
ATP	0.13	1.80	1.09	1.40	8.39	0.78	100	100
dATP	0.16	3.80	1.27	1.70	7.94	0.45	95	58
Poly(P)4	1.04	1.60	1.58	1.60	1.52	0.94	18	121
NAD (*+ATP*) [a]	0.83	0.90	1.76	1.20	1.76	1.33	20	171
NAD (*+poly(P)*) [b]	0.58	2.90	2.55	1.80	2.55	0.62	30	79

Kinetic constants were calculated with Lineweaver-Burk plot. Each of 0.017 units (determined in the presence of 5.0 mM ATP) of Mfnk and Ppnk was used for each assay. [a] Kinetic constants for NAD in the presence of 5.0 mM ATP. [b] Kinetic constants for NAD in the presence of 5.0 mM poly(P)4.

TABLE 4 Kinetic constants of YfjB and Ppnk

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m	
	ATP	NAD	ATP	NAD	ATP	NAD
YfjB	2.50	2.00	55.0	125.0	22.0	38.0
Ppnk	1.80	0.90	2.72	2.33	1.51	1.20

TABLE 5 Effects of metal ions on poly(P)- and ATP-dependent NAD kinase activities of Mfnk and Ppnk

Metal	Poly(P)-dependent activity (%)		ATP-dependent activity (%)	
	Mfnk	Ppnk	Mfnk	Ppnk
None	nd	nd	nd	nd
$MgCl_2$	100	100	100	100
$MnCl_2$	143	268	136	246
$ZnCl_2$	30	30	51	51
$CaCl_2$	65	34	61	39
$CoCl_2$	51	55	28	29
$FeCl_2$	nd	nd	nd	nd
$CuCl_2$	nd	8	nd	25
NaCl	nd	nd	nd	nd
KCl	nd	nd	nd	nd
LiCl	nd	nd	nd	nd

Poly(P)- and ATP-dependent NAD kinase activities were assayed in the reaction mixture described in MATERIALS AND METHODS, in which 5.0 mM $MgCl_2$ was replaced for each of 1.0 mM metal ions listed above. Each of poly(P)- and ATP-dependent NAD kinase activities of Mfnk and Ppnk in the presence of 1.0 mM $MgCl_2$ was relatively taken as 100 %. nd, not detected.

TABLE 6 Effects of NADP, NADH, and NADPH on poly(P)- and ATP-dependent NAD kinase activities of Mfnk and Ppnk

Compounds	Conc. (mM)	ATP-dependent activity (%)		Poly(P)-dependent activity (%)	
		Mfnk	Ppnk	Mfnk	Ppnk
None	0	100	100	100	100
NADPH	0.02	100	84	100	100
	0.05	66	71	50	92
	0.50	nd	6	nd	28
NADH	0.05	100	100	100	96
	0.50	66	89	50	76
	1.00	nd	76	nd	64
NADP	0.50	100	100	100	100

Poly(P)- and ATP-dependent NAD kinase activities were assayed in the reaction mixture described in MATERIALS AND METHODS containing each of NADP, NADH, and NADPH at indicated concentrations. Each of poly(P)- and ATP-dependent NAD kinase activities of Mfnk and Ppnk in the absence of NADP, NADH, and NADPH was relatively taken as 100 %. nd, not detected.

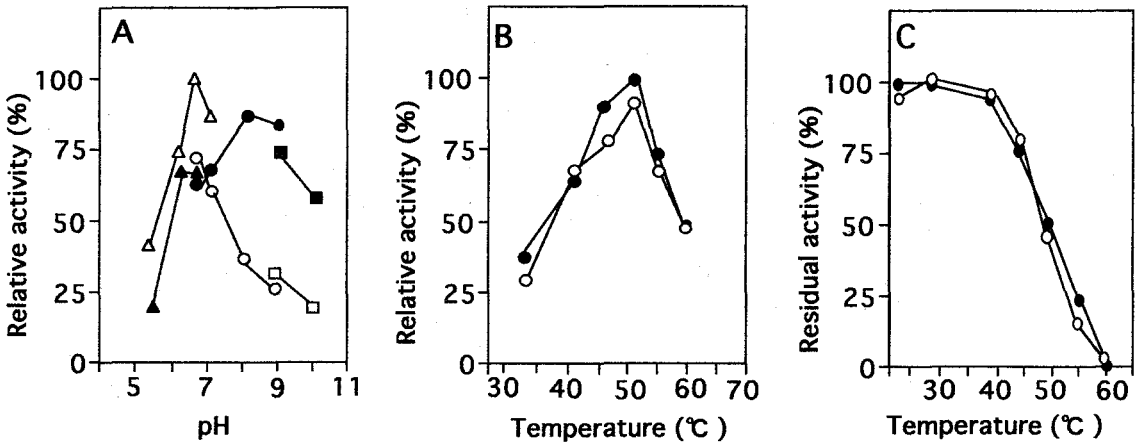

FIG. 3 Effects of pH and temperature on poly(P)- and ATP-dependent NAD kinase activities of poly(P)/ATP-NAD kinase (Ppnk). (A) Effects of pH on poly(P) (△, ○, □)- and ATP (▲, ●, ■)-dependent NAD kinase activities. NAD kinase activities were assayed as described in MATERIALS AND METHODS with each of 100 mM sodium acetate (△, ▲), Tris-HCl (○, ●), and glycine-NaOH (□, ■). (B) Effects of temperature on poly(P) (○)- and ATP (●)-dependent NAD kinase activities. NAD kinase activities were assayed as described in MATERIALS AND METHODS at each of the indicated temperatures. (C) Thermal stability of the poly(P)/ATP-NAD kinase. The purified enzyme was incubated for 10 min at each of the indicated temperatures and then the residual poly(P) (○)- and ATP (●)-dependent NAD kinase activities were assayed as described in MATERIALS AND METHODS.

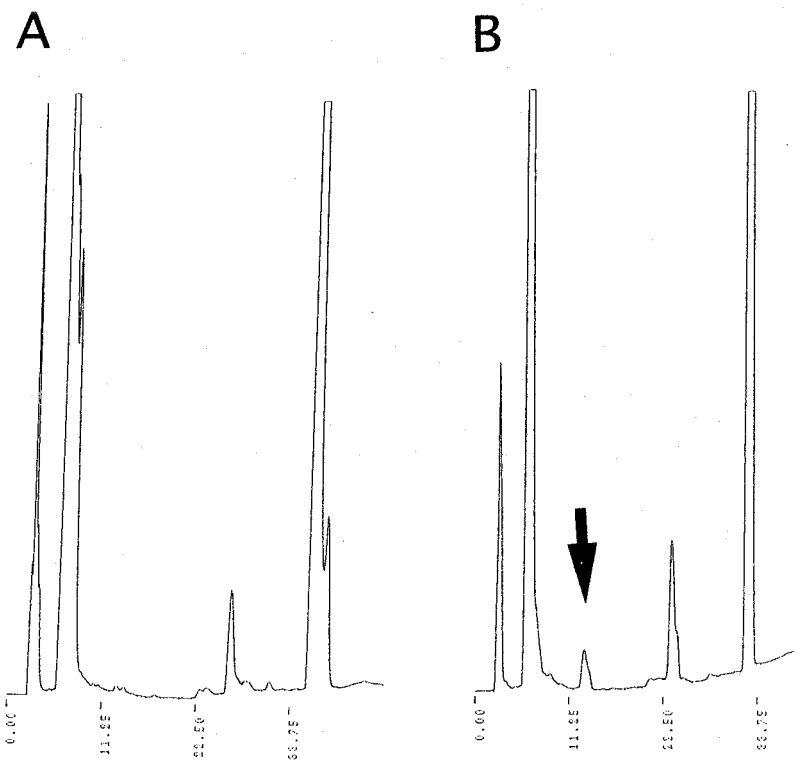

FIG. 4 Assay of NADH kinase activity. ATP-dependent NADH kinase reactions were performed for 30 min as described in MATERIALS AND METHODS in the absence (A) and presence (B) of NAD kinase (Ppnk). Arrow indicates the formed NADPH.

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Section 2 ATP-NAD kinase of *Saccharomyces cerevisiae*

As described in previous Section, primary structure of Utr1p (GenBank: L26347-2, Z49549-1; SwissProt: P21373), encoded by *UTR1* (ORF ID: YJR049c) of *Saccharomyces cerevisiae* shows homologies with N-terminal and internal amino acid sequences of inorganic polyphosphate [poly(P)]/ATP-NAD kinase (Mfnk) of *Micrococcus flavus*. Although the involvement of Utr1p in the ferrireductase system of *S. cerevisiae* (1) is suggested, no definitive conclusions as to the intrinsic function of Utr1p have been obtained. In the study described in this chapter, Utr1p was shown to be an ATP-NAD kinase and characterized. Furthermore, properties of NAD kinases studied in this thesis are compared and discussed.

MATERIALS AND METHODS

Bacterial strains. *S. cerevisiae* ATCC7754 was cultured in YPD medium (2). As a host for plasmid amplification, *Escherichia coli* DH5α (Toyobo, Osaka, Japan) was routinely cultured at 37 ℃ in Luria-Bertani (LB) medium (2) supplemented with ampicillin (100 μg/ml). The growth conditions for the derivative strains of *E. coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany) are described in the text.

Assays. NAD kinase activity (ATP-dependent NAD kinase activity) was assayed by means of a two-step method as described (3) except that the reaction was performed at 30 ℃. Details are described in Section 1 of Chapter I. NADH kinase activity was assayed as described in Section 1 of Chapter I. Kinetic constants were calculated with Lineweaver-Burk plot. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as a standard.

Cloning of *UTR1*. *UTR1* was amplified by PCR (Takara Biomedicals, Kyoto, Japan) from the genomic DNA isolated from *S. cerevisiae* ATCC7754 by the method as described (2). PCR was performed in a reaction mixture (100 μl) consisting of 2.5 units KOD polymerase (Toyobo, Osaka, Japan), 0.25 μg genomic DNA, 40 pmol *UTR1 Nco*I primer 5' TGCCATGGAGGAGAATGACATGAATAATGGCGT 3', 40 pmol *UTR1 Bam*HI primer 5' ATGGATCCTTATACTGAAAACCTTGCTTGAGAAG 3', 20 nmol dNTPs, 100 nmol $MgCl_2$, 8.0 % dimethyl sulfoxide, and the reaction buffer #1 supplemented with the KOD polymerase. The cycle condition was as follows; 98 ℃ 15 sec, 60 ℃ 2 sec, 74 ℃ 30 sec,

25 cycles. The 1.59 kb PCR product, of which nucleotide sequence was determined and identified to be *UTR1*, was digested with *Nco*I and *Bam*HI, and ligated into pET-14b (Novagen) digested with *Nco*I and *Bam*HI, which yielded pSK41. Derivative strains, SK41 and SK45 were obtained through the transformation of *E. coli* BL21(DE3)pLysS with pSK41 and pET-14b, respectively.

Expression of Utr1p in *E. coli*. For the expression of Utr1p, SK41 was inoculated into 480 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin and 34 μg/ml chroramphenicol, and then cultured at 37 ℃ aerobically until A_{600} reached 1.0. The culture was then transferred to 13.5 l of the same medium with the same antibiotics and cultivation was continued at 37 ℃ aerobically for 2 h until A_{600} reached 0.70; then isopropyl-β-D-thiogalactopyranoside was added to give a final concentration of 0.10 mM, and the cultivation was continued further at 16 ℃ aerobically for 36 h. As a control, SK45 in a 10 ml culture was also treated by the same manner.

Purification of Utr1p. Centrifugation was carried out at 20,000 x g, 4 ℃ for 20 min, and dialysis was at 4 ℃ overnight against KEG (10 mM potassium phosphate (pH 7.0), 1.0 mM EDTA, 10 % glycerol, and 10 mM 2-mercaptoethanol), unless otherwise stated. Utr1p was purified by measuring ATP-dependent NAD kinase activity as an index. The cells of SK41 (59 g wet wt.) expressing Utr1p were collected, suspended in 100 ml of KEG, and then disrupted with Sonifire (Branson, Danbury, CT). Cell extract, after centrifugation, was supplemented with 1.0 mM phenylmethylsulfonyl fluoride and Utr1p was precipitated with ammonium sulfate (0 – 30 %). The precipitate was dissolved in KEG, dialyzed, applied onto a DEAE-Toyopearl 650M column (2.8 x 22 cm) (Tosoh, Tokyo, Japan) equilibrated with KEG, and proteins were eluted with a linear gradient of NaCl (0 – 600 mM, 500 ml) in 10 ml fractions every 4.0 min. Fractions with Utr1p, which were obtained by elution with 300 – 500 mM NaCl, were combined, dialyzed against KEG containing 10 mM MgCl2, and loaded onto an AF-Blue Toyopearl 650 ML column (2.8 x 7.0 cm) (Tosoh) equilibrated with KEG containing 10 mM MgCl2. Absorbed proteins were eluted with a linear gradient of NaCl (0 - 3.0 M, 200 ml) in 5.0 ml fractions every 5.0 min. Fractions with Utr1p, which were obtained by elution with 2.1 – 3.0 M NaCl, were pooled and concentrated to 1.5 ml by an ultrafiltration with an Amicon model 8200 (Amicon, Beverly, MA). The concentrate was loaded onto a Superdex pg200 column (1.5 x 117 cm) (Amersham Pharmacia Biotech, Buckinghamshire, England) equilibrated with 10 mM potassium phosphate (pH 7.0) containing 150 mM NaCl, and eluted with the same buffer in 2.0 ml fractions every 4.0 min. The fractions (nos. 49 -

51) containing Utr1p were combined, saturated with ammonium sulfate (10 %), and applied onto a Butyl-Toyopearl 650M column (0.80 x 1.0 cm) (Tosoh) equilibrated with KEG containing 10 % ammonium sulfate. Proteins were eluted with a linear gradient of ammonium sulfate (10 – 0 %, 10 ml) in 500 μl fractions every 15 s. The fractions with Utr1p, which were obtained by elution with 5.0 – 3.0 % ammonium sulfate, were combined, dialyzed against 10 mM potassium phosphate (pH 7.0), and served as a purified Utr1p.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5 % polyacrylamide gel as described (5). Proteins in the gel were visualized with Coomassie Brilliant Blue R-250. The molecular mass of Utr1p was determined by gel filtration chromatography on a Superdex pg200 column (1.5 x 117 cm). The purified Utr1p was loaded onto the column and eluted with 10 mM potassium phosphate (pH 7.0) containing 150 mM NaCl in 2.0 ml fractions every 4.0 min. Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) was used as standard proteins. N-terminal amino acid sequence was analyzed with a Procise 492 protein sequence system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA). DNA sequence was determined with an automated DNA sequencer (Model 377; Applied Biosystems Division of Perkin-Elmer). Blast (6) on the World-Wide Web site of the DNA Databank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html) was used for the homology search. Sequence data were obtained from the GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) and SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today) databases.

RESULTS

Identification of *UTR1* as ATP-NAD kinase gene. As a consequence of homology search, it was shown that the amino acid sequence of poly(P)/ATP-NAD kinase (Mfnk) of *M. flavus* exhibited a significant homologies with many hypothetical proteins including *S. cerevisiae* Utr1p (GenBank: L26347-2, Z49549-1; SwissProt: P21373) (1, 7, 8). Utr1p is a polypeptide of 530 amino acids with a calculated molecular mass of 59,469 Da, and encoded by *UTR1* (ORF ID: YJR049c) consisting of 1,593 nucleotides (7) (Fig. 1). *UTR1* cloned from genomic DNA of *S. cerevisiae* was expressed in SK41 as described in MATERIALS

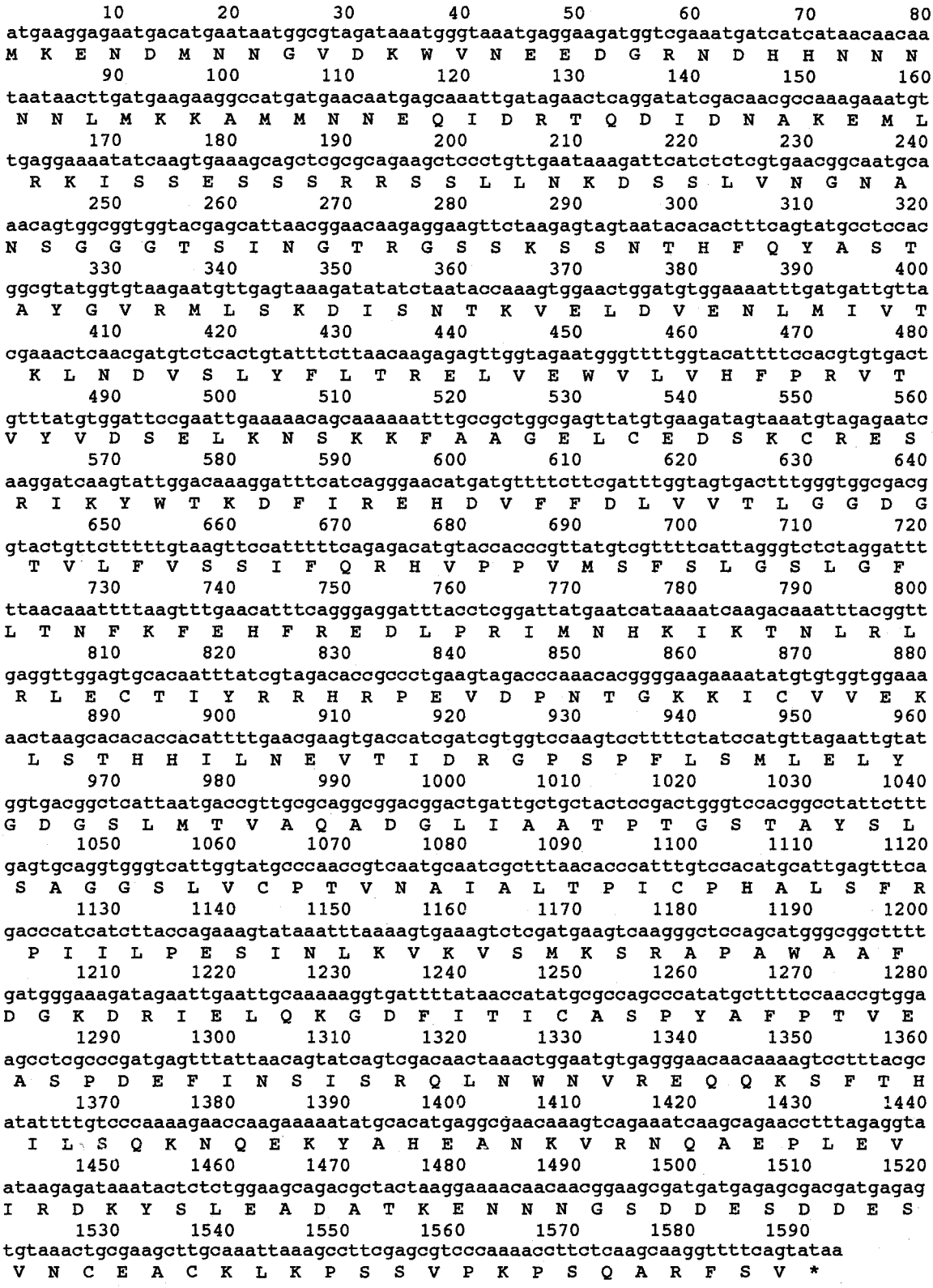

FIG. 1 Nucleotide sequence of *UTR1*. Deduced amino acid sequence of Utr1p is indicated below nucleotide sequence.

AND METHODS. Cell extracts of SK41 showed much higher ATP-dependent NAD kinase activity (0.094 units/mg) than that of control strain SK45 (0.0042 units/mg). The result strongly indicated that *UTR1* is the gene for NAD kinase. Utr1p purified from the cell extract of SK41 (Table 1) migrated as a single protein band with a molecular mass of 60 kDa on SDS-PAGE (Fig. 2), and was eluted as a single peak of a 360 kDa protein on gel filtration chromatography (data not shown). Furthermore, N-terminal amino acid sequence, ^{1}MEENDMNN^{8}, of the 60 kDa protein was identical with that deduced from *UTR1*. The purified Utr1p utilized ATP, but not poly(P) as phosphoryl donors (Table 1). Based on the results described above, *UTR1* is concluded to be a gene for ATP-NAD kinase consisting of six identical subunits with 60 kDa.

TABLE 1 Purification of Utr1p from SK41

Step	Total protein (mg)	Total activity (units)	Activity yield (%)	Specific activity (units/mg)	Purification (fold)
Cell extract	5,390	508	100	0.094	1.0
Ammonium sulfate	2,496	235	46.0	0.094	1.0
DEAE-Toyopearl	940	323	64.0	0.340	3.6
AF-Blue-Toyopearl	30.8	82.7	16.0	2.69	28.6
Superdex gp200	1.30	11.8	0.40	9.08	96.6
Butyl-Toyopearl	0.22	3.57	0.07	16.20	172

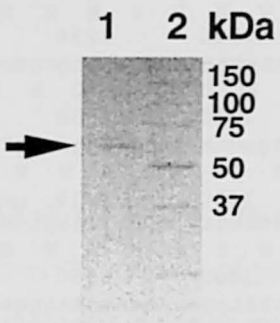

FIG. 2 SDS-PAGE of Utr1p. Lane 1: Purified Utr1p (3.0 μg). Lane 2: Protein markers (Bio-Rad Laboratories, Hercules, CA). Utr1p is indicated by an arrow.

Characterization of Utr1p. Properties of Utr1p were compared with those of *E. coli* ATP-NAD kinase (YfjB). Utr1p and YfjB utilized ATP and other nucleoside triphosphates, although Utr1p did not use UTP, GTP, and TTP (Table 2). Nucleoside diphosphates,

glucose-6-phosphate, *p*-nitrophenylphosphate, and other energy-rich compounds (phosphocreatine and phosphoenolpyruvate) were also inert for both YfjB and Utr1p as phosphoryl donors (Table 2). Utilization of poly(P) by Utr1p was not detected (Table 2), although a little utilization by YfjB was detected. Kinetic constants of Utr1p were as follows; K_m and V_{max} values were 0.50 mM, 1.20 μM/(min ·unit) for NAD, and 0.60 mM, 1.00 μM/(min · unit) for ATP, respectively. Optimum pH of Utr1p was 8.0 (Fig. 3A) and optimum temperature was 30 ℃ (Fig. 3B). Half of the activity of Utr1p was lost on treatment at 37 ℃ for 10 min (Fig. 3C). Bivalent metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} were indispensably required for the activity of Utr1p and YfjB (Table 3). Mn^{2+} was most effective activator for Utr1p and YfjB. *p*-Chloromercuribenzoate and HgCl2 inhibited the activity of Utr1p (Table 4). Dithiothreitol, 2-mercaptetanol, and reduced glutathione gave no effect on Utr1p at 1.0 mM. NADP, NADH and NADPH inhibited YfjB and Utr1p, while YfjB was more effectively inhibited by NADH and NADPH at low concentrations (Table 4). In accordance with this fact, Utr1p showed ATP-dependent NADH kinase activity that was confirmed by TLC (data not shown) and HPLC (Fig. 4) analyses. NADH kinase activity was 46 % to NAD kinase one.

TABLE 2 Phosphoryl donor specificities of YfjB and Utr1p

Phosphoryl donor	Relative activity (%)	
	YfjB	Utr1p
UTP	109	nd
ATP	100	100
CTP	60	67
GTP	56	nd
dATP	42	39
TTP	40	nd
ADP	nd	nd
AMP	nd	nd
Phosphocreatine	nd	nd
Phosphoenolpyruvate	nd	nd
p-Nitrophenylphosphate	nd	nd
Glucose-6-phosphate	nd	nd
Metaphosphate	2.4	nd

NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of the phosphoryl donors listed above. Metaphosphate was obtained from Wako Pure Chemical Industries (Osaka, Japan), and was used at 1.0 mg/ml as poly(P). Other phosphoryl donors were purchased from Sigma (St. Louis, MO) and used at 5.0 mM. Each of activity of YfjB and Utr1p for 5.0 mM ATP was taken as 100 %. nd, not detected.

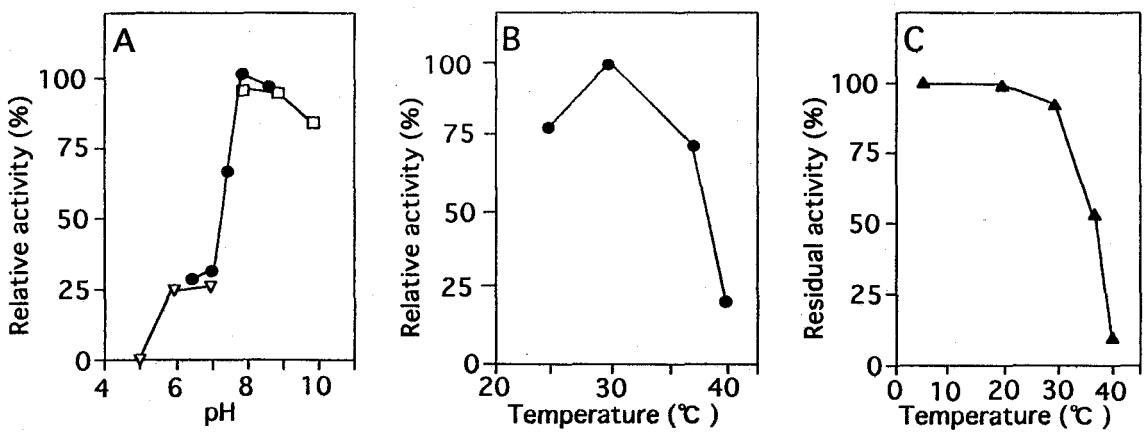

FIG. 3 Effects of pH and temperature on ATP-dependent NAD kinase activity of Utr1p. (A) Effect of pH on ATP-dependent NAD kinase activity of Utr1p. NAD kinase activity was assayed as described in MATERIALS AND METHODS with each of 100 mM sodium acetate (▲), Tris-HCl (●), and glycine-NaOH (□). (B) Effect of temperature on ATP-dependent NAD kinase activity of Utr1p. NAD kinase activity was assayed as described in MATERIALS AND METHODS at each of the indicated temperatures. (C) Thermal stabilitiy of Utr1p. The purified Utr1p was incubated for 10 min at each of the indicated temperatures and then the residual activity was assayed as described in MATERIALS AND METHODS.

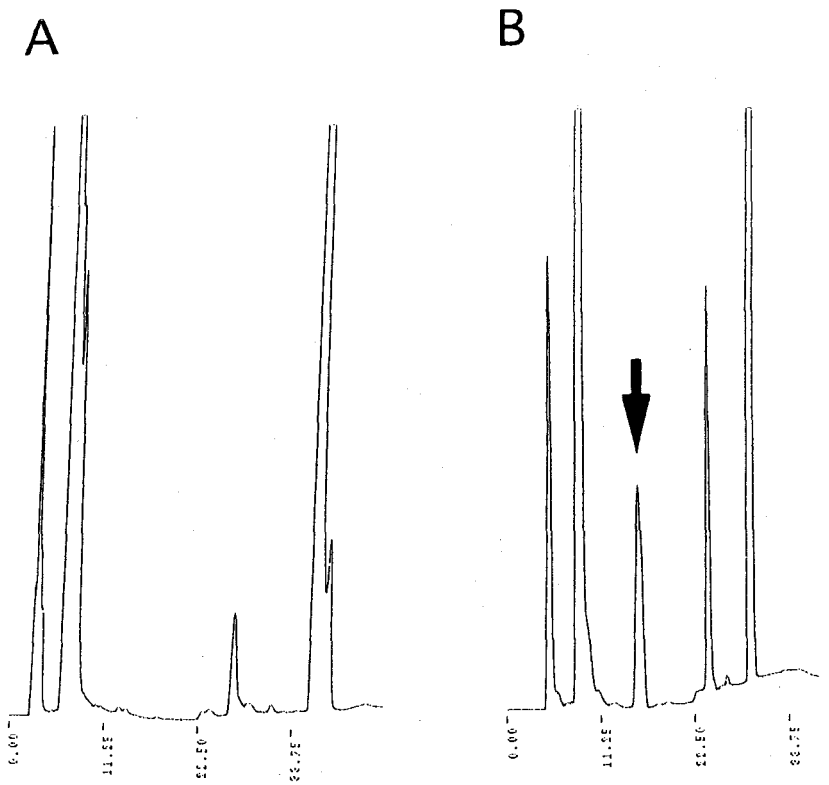

Fig. 4 Assay of NADH kinase activity. ATP-dependent NADH kinase reactions were performed for 30 min as described in MATERIALS AND METHODS in the absence (A) and presence (B) of NAD kinase. Arrow indicates the formed NADPH.

TABLE 3 Effects of metal ions on ATP-dependent activities of YfjB and Utr1p

Metal	Relative activity (%) YfjB	Utr1p
None	nd	nd
$MgCl_2$	100	100
$MnCl_2$	242	221
$ZnCl_2$	104	179
$CaCl_2$	85	29
$CoCl_2$	73	ND
$FeCl_2$	nd	nd
$CuCl_2$	nd	nd
NaCl	nd	nd
KCl	nd	nd
LiCl	nd	nd

ATP-dependent NAD kinase activity was assayed in the reaction mixture described in MATERIALS AND METHODS, in which 5.0 mM $MgCl_2$ was replaced for each of 1.0 mM metal ions listed above. Each of activity of YfjB and Utr1p in the presence of 1.0 mM $MgCl_2$ was relatively taken as 100 %. nd, not detected. ND, not determined.

TABLE 4 Effects of various compounds on the ATP-dependent NAD kinase activities of YfjB and Utr1p

Compound	Conc. (mM)	Relative activity (%) YfjB	Utr1p
None	0	100	100
NADPH	0.005	58	92
	0.010	24	80
	0.050	ND	57
NADH	0.005	68	100
	0.010	39	100
	0.050	ND	83
NADP	0.050	83	61
	0.100	79	ND
p-Chloromercuribenzoate	1.0	nd	12
$HgCl_2$	1.0	nd	50

ATP-dependent NAD kinase activity was assayed in the reaction mixture described in MATERIALS AND METHODS containing each of various compounds listed above at the indicated concentrations. When each of *p*-chloromercuribenzoate and $HgCl_2$, inhibitors for isocitrate dehydrogenase, was present in the reaction mixture, NADP was enzymatically determined with glucose-6-phosphate dehydrogenase. Activity in the absence of the compounds was taken as 100 %. nd, not detected. ND, not determined.

DISCUSSION

In this study, *UTR1* (ORF ID: YJR049c) of *S. cerevisiae,* which was initially reported as an *U*nidentified *TR*anscript of unknown function (7) and later indicated to be a component of the ferrireductase system of *S. cerevisiae* (1), was identified to be a gene of ATP-NAD kinase. The ferrireductase system of this organism is required for the reduction of extracellular ferric chelates to their ferrous counterparts and consists of three components, Fre1p, NADPH dehydrogenase, and Utr1p (1). This fact is compatible with the results showing that Utr1p is ATP-NAD kinase and that Utr1p also shows NADH kinase activity. Utr1p should contribute to the ferrireductase system through the supply of NADP or NADPH and therefore may regulate this system through the regulation of NADP and NADPH levels. The fact that Utr1p exhibits NADH kinase activity was in contradiction with the report by Iwahashi *et al.* that in *S. cerevisiae* NADH-specific kinase was localized exclusively in the mitochondria, and NAD-specific kinase was distributed in the microsomal and cytosol fractions but not in the mitochondria (9). Thus, the NADH kinase activity of Utr1p will give a new insight into the regulation of NADP in *S. cerevisiae*. Subunit structure of Utr1p [hexamer of 60 kDa subunit] is apparently different from that of NAD kinase previously purified from *S. cerevisiae* by Tseng (10) [homotetramer of 31 kDa subunit]. In *S. cerevisiae*, Utr1p may be possibly cleaved or modified and result in a homotetramer of 31 kDa subunit like NAD kinase reported by him. On the other hand, Teng's NAD kinase may be encoded by other putative NAD kinase genes of *S. cerevisiae*, *POS5* (GenBank: Z73544-1, X84260-1, SwissProt: Q06892, ORF ID: YPL188w) or YEL041w (GenBank: U18779-13, SwissProt: P32622) of which primary structures also showed homology with that of Utr1p.

Thus, in this thesis properties were revealed of two ATP-NAD kinases [YfjB of *E. coli* (Chapter II) and Utr1p of *S. cerevisiae* (this Section)] and two poly(P)/ATP-NAD kinases [Mfnk of *M. flavus* (Chapter I) and Ppnk of *Mycobacterium tuberculosis* H37Rv (Section 1 of this Chapter)]. Properties of these NAD kinases (YfjB, Utr1p, Mfnk, and Ppnk) are compared (Table 5-8) and are discussed from the following standpoints.

1. *Phosphoryl donor specificity.* YfjB, Utr1p, Mfnk, and Ppnk utilized ATP and other nucleoside triphosphates, but not nucleoside di- and mono-phosphates, other energy-rich compounds, and *p*-nitrophenylphosphate. However, the abilities of YfjB and Utr1p to utilize poly(P) were significantly lower than those of Mfnk and Ppnk (Table 5). Thus, YfjB and Utr1p were named "ATP-NAD kinase", and Mfnk and Ppnk were

"poly(P)/ATP-NAD kinase", as emphasized throughout this thesis.

2. *Phosphoryl acceptor specificity.* Utr1p, Mfnk, and Ppnk showed NADH kinase activities, while YfjB not. No NADH kinase activity of YfjB is in accordance with the fact that NADH strongly inhibits YfjB and is allosteric regulator for YfjB, while NADH inhibited Utr1p, Mfnk, and Ppnk with lower efficiencies than YfjB (Table 6). These facts indicate that mechanisms of NADP and NADPH syntheses show diversity in microbes.
3. *Metal ion requirements.* All these enzymes required bivalent metal ions such as Mn^{2+} and Mg^{2+} (Table 7). Mn^{2+} seems to be an more effective activator for all these enzymes than Mg^{2+}.
4. *Inhibitions by SH reagents.* All these enzymes were inhibited by SH reagents (*p*-chloromercuribenzoate and HgCl2) (Table 6), suggesting SH groups of all these enzymes play important roles for their activities.
5. *Optimum pH, temperature and thermal stability.* When optimum pH of these enzymes were compared, there is a tendency that ATP-dependent NAD kinase activities of Utr1p and Ppnk seem to prefer alkaline condition. It should be noted that among NAD kinase activities of these enzymes, only poly(P)-dependent activity of Ppnk prefers acidic condition (pH 6.5). This preference for acidic condition will show advantages to produce NADP in industrial scale that is discussed in Chapter IV, since NADP is more stable in acidic condition. Utr1p notably shows lower thermal stability than YfjB, Mfnk and Ppnk (Table 8).

TABLE 5 Phosphoryl donor specificities of YfjB, Utr1p, Mfnk, and Ppnk

Phosphoryl donor	Relative activity (%)			
	YfjB	Utr1p	Mfnk	Ppnk
UTP	109	nd	87	88
ATP	100	100	100	100
CTP	60	67	73	16
GTP	56	nd	88	47
dATP	42	39	91	96
TTP	40	nd	74	33
ADP	nd	nd	nd	nd
AMP	nd	nd	nd	nd
Phosphocreatine	nd	nd	ND	ND
Phosphoenolpyruvate	nd	nd	nd	nd
p-Nitrophenylphosphate	nd	nd	nd	nd
Glucose-6-phosphate	nd	nd	nd	nd
Metaphosphate	2.4	nd	88	178

Each of activity of enzymes for 5.0 mM ATP was relatively taken as 100 %. nd, not detected. ND, not determined.

TABLE 6 Effects of various compounds on activities of YfjB, Utr1p, Mfnk, and Ppnk

Compound	Conc. (mM)	Relative activity (%)			
		YfjB	Utr1p	Mfnk	Ppnk
None	0	100	100	100	100
NADPH	0.005	58	92	100	100
	0.010	24	80	100	100
	0.020	ND	ND	100	84
	0.050	ND	48	66	71
NADH	0.005	68	100	100	100
	0.010	39	100	100	100
	0.050	ND	83	100	100
	0.500	ND	ND	66	89
NADP	0.050	83	61	100	100
	0.100	79	ND	100	100
p-Chloromercuribenzoate	1.0	nd	12	nd	nd
HgCl2	1.0	nd	50	nd	nd

Each of activities of enzymes in the absence of NADP, NADH, and NADPH was relatively taken as 100 %. nd, not detected. ND, not determined.

TABLE 7 Effects of metal ions on activities of YfjB, Utr1p, Mfnk, and Ppnk

Metal	Relative activity (%)					
	YfjB	Utr1p	Mfnk		Ppnk	
			(P)	(A)	(P)	(A)
None	nd	nd	nd	nd	nd	nd
$MgCl_2$	100	100	100	100	100	100
$MnCl_2$	242	221	143	136	168	246
$ZnCl_2$	104	179	30	51	30	51
$CaCl_2$	85	29	65	61	34	39
$CoCl_2$	73	ND	51	28	55	29
$FeCl_2$	nd	nd	nd	nd	nd	nd
$CuCl_2$	nd	nd	nd	nd	8	25
NaCl	nd	nd	nd	nd	nd	nd
KCl	nd	nd	nd	nd	nd	nd
LiCl	nd	nd	nd	nd	nd	nd

Each of activities of enzymes in the presence of 1.0 mM $MgCl_2$ was relatively taken as 100 %. Poly(P)- and ATP-dependent NAD kinase activities are presented by (P) and (A), respectively. nd, not detected. ND, not determined.

TABLE 8 Optimum pH, optimum temperature, and thermal stabilities of Utr1p, YfjB, Mfnk, and Ppnk

	YfjB	Utr1p	Mfnk	Ppnk
Optimum pH	7.5	8.0 – 9.0	7.0 (A, P)[b]	6.5 (P) 8.0-9.0 (A)
Optimum temperature (℃)	60	30	55 (P, A)[b]	50 (P, A)
Thermal stability (℃)[a]	65	37	60 (P, A)	50 (P, A)

[a] Temperature required to decrease half of the activity of each NAD kinase. [b] pH and temperatures for poly(P)-, ATP-, and both of poly(P)- and ATP-dependent NAD kinase activities are presented by (P), (A), and (P, A), respectively.

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Section 3 Molecular structure of NAD kinases

As described in previous Chapters and Sections, primary structures and other properties of poly(P)/ATP-NAD kinases of *Micrococcus flavus* (Mfnk) and *Mycobacterium tuberculosis* H37Rv (Ppnk) and of ATP-NAD kinases of *Escherichia coli* (YfjB) and *Saccharomyces cerevisiae* (Utr1p) were determined. In this Section, attention was focused on the structures of these enzymes to deepen insight as to the structure of NAD kinase.

MATERIALS AND METHODS

Site directed mutagenesis. Site directed mutagenesis was performed with QuickChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA). Derivative plasmid of pET-14b (Novagen, Darmstadt, Germany), pSK67 (Section 2 in Chapter II) was used as a template. Primers listed in Table 1 were used.

TABLE 1 Primers used for the site-directed mutagenesis

YfjB E36A	gtgcacaaaaggttacgcggtcatcgttgagc
YfjB E36A RC	gctcaacgatgaccgcgtaacctttgtgcac
YfjB E163V	gcgcatatgattgagttcgtagtgtatatcgacgag
YfjB E163V RC	ctcgtcgatatacactacgaactcaatcatatgcgc
YfjB R232D	cacgatccgtctggatttttcgcatcgccgtaacgac
YfjB R232D RC	gtcgttacggcgatgcgaaaaatccagacggatcgtg
YfjB L259E	ggaaggtgaagatgtcgagattcgtcgctgtgattac
YfjB L259E RC	gtaatcacagcgacgaatctcgacatcttcaccttcc

Other methods. Blast (1) on World-Wide Web site (http://www.ddbj.nig.ac.jp/ Welcome-j.html) was used for homology search. Sequences were obtained from GenBank (http://www.genome.ad.jp/dbget-bin/www_bfind?genbank-today) or SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today). Alignment of the sequences was performed with CLUSTAL W (1.8) (2) on the World-Wide Web site of DNA Databank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html).

RESULTS AND DISCUSSION

Subunit structures of NAD kinases. In this thesis, subunit structures of four NAD kinases (Mfnk, Ppnk, YfjB, and Utr1p) were determined; Mfnk, 34 kDa (dimer), Ppnk, 35 kDa (tetramer), YfjB, 30 kDa (hexamer), and Utr1p, 60 kDa (hexamer). They are, except for Utr1p, comparable with those of other NAD kinases purified so far, pigeon liver, 34 kDa (octamer) (3), *S. cerevisiae*, 31 kDa (tetramer) (4), and *C. utilis,* 32 kDa (octamer) (5) (Fig. 1), indicating NAD kinases are generally multimer consisting of subunits with 30-35 kDa. Subunit structure of Utr1p [hexamer of 60 kDa subunit] is apparently different from that of NAD kinase previously purified from *S. cerevisiae* by Tseng (4) [homotetramer of 31 kDa subunit]. In *S. cerevisiae*, Utr1p may be possibly cleaved or modified and result in a homotetramer of 31 kDa subunit like NAD kinase reported by Tseng.

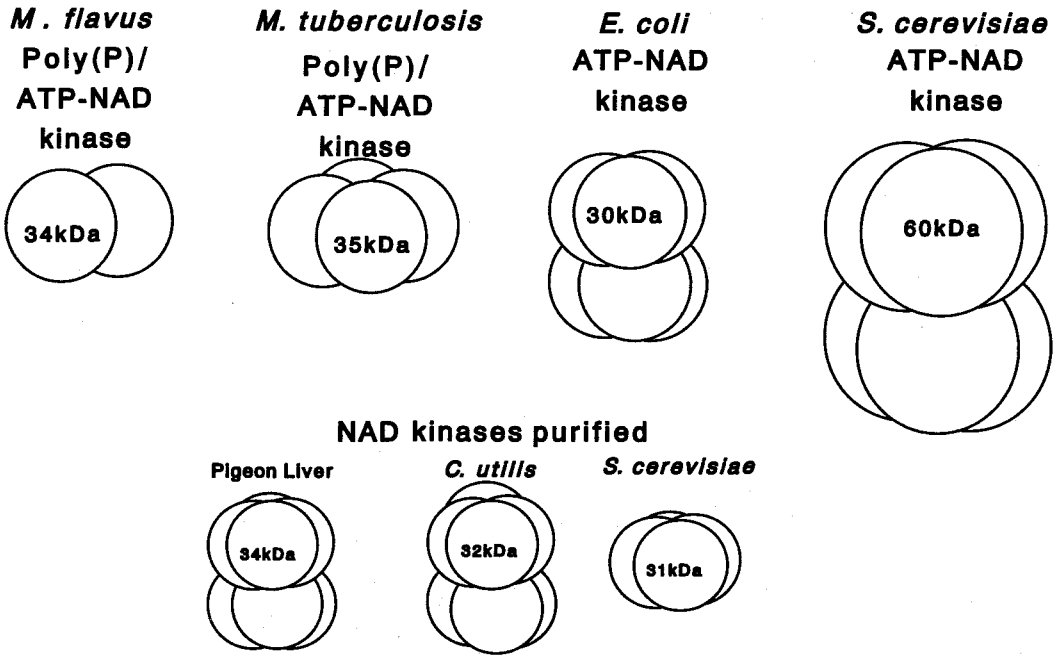

FIG. 1 Subunit structures of NAD kinases purified.

Primary structures of other NAD kinase homologues. As a result of Blast search, primary structure of Mfnk showed homologies with those of many "probable poly(P)/ATP-NAD kinases" including Ppnk, YfjB, and Utr1p (Table 2). Although these probable proteins were deposited as hypothetical or function-unknown proteins, they would be renamed after identification of Ppnk. Other than Utr1p, two NAD kinase homologues of *S. cerevisiae* (Q06892 and P32622) were found, while only one NAD kinase homologue of human

(O95544) was found. Other features of these homologues are listed in Table 3. Alignment of primary structures of these probable proteins indicates that significant conserved sequences are not found, although some amino acid residues are conserved (Fig. 2).

Primary structures of Mfnk, Ppnk, YfjB, and Utr1p. Then, alignment of primary structures of Mfnk, Ppnk, YfjB, and Utr1p was performed (Fig. 3). In spite of the significant difference of phosphoryl donor and acceptor specificities, primary structures of Mfnk, Ppnk, YfjB, and Utr1p were highly conserved. This conservation indicates that molecular evolution of NAD kinase, acquirement or loss of the ability to utilize NADH and especially poly(P) in an evolutionary process, should occur as a result of accumulation of point mutations with limited numbers.

Determination of amino acid residues responsible for poly(P) utilization. Although primary structures of poly(P)/ATP- and ATP-NAD kinases are highly conserved, some amino acid residues are distinguishable between the two NAD kinases (Fig. 4) and they were supposed those that determined the ability to utilize poly(P). In other words, they are supposed to be those mutated in a molecular evolutionary process of NAD kinase as discussed above. To confirm this supposition, these amino acid residues of YfjB (E36, E163, R232, and L259) were converted to those of poly(P)/ATP-NAD kinases with site directed mutagenesis techniques. However, conversion of each or all these amino acid residues of YfjB to those of poly(P)/ATP-NAD kinases did not affect the poly(P) utilizing ability of YfjB (Table 4).

In order to understand the evolutionary process from a standpoint of structural biology, or in other words, to identify the amino acid residues for poly(P) utilization, determination of three dimensional structures of poly(P)/ATP- and ATP-NAD kinases and comparison of them are indispensable. Identification of the amino acid residues for poly(P) utilization will lead to an establishment of biotechnological techniques to give other ATP utilizing enzymes the ability to utilize poly(P). Since poly(P) is commercially obtainable in large quantities at extremely lower cost than ATP, such techniques will make possible to utilize poly(P) instead of ATP in the industrial production that requires ATP.

In the next Section, crystallization and preliminary X-ray analysis of poly(P)/ATP-NAD kinase is described. In Chapter IV, advantages of utilizing poly(P) in the industrial production will be described.

TABLE 2 Proteins in SwissProt showing homology with Mfnk

Sequences producing significant alignments:	(bits)	Value
sp|Q9S219|PPNK_STRCO PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	288	1e-77
sp|O33196|PPNK_MYCTU INORGANIC POLYPHOSPHATE/ATP-NAD KINASE (EC...	244	2e-64
sp|Q49897|PPNK_MYCLE INORGANIC POLYPHOSPHATE/ATP-NAD KINASE (EC...	241	2e-63
sp|Q9KTP8|PPNK_VIBCH PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	116	8e-26
sp|Q9HZC0|PPNK_PSEAE PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	111	2e-24
sp|O26958|PPNK_METTH PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	111	3e-24
sp|Q9CNU2|PPNK_PASMU PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	109	1e-23
sp|Q51841|PPNK_PORGI PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	109	1e-23
sp|O83455|PPNK_TREPA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	107	4e-23
sp|O67055|PPNK_AQUAE PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	106	7e-23
sp|P44497|PPNK_HAEIN PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	106	1e-22
sp|Q58327|PPNK_METJA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	104	3e-22
sp|O51291|PPNK_BORBU PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	104	3e-22
sp|P74430|PPN1_SYNY3 PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	102	1e-21
sp|Q9JQL9|PPNK_NEIMA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	100	6e-21
sp|P73955|PPN2_SYNY3 PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	95	2e-19
sp|P37768|PPNK_ECOLI PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	95	2e-19
sp|P58057|PPNK_ECO57 PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	94	4e-19
sp|O58801|PPNK_PYRHO INORGANIC POLYPHOSPHATE/ATP-NAD KINASE (EC...	93	8e-19
sp|Q9V081|PPNK_PYRAB INORGANIC POLYPHOSPHATE/ATP-NAD KINASE (EC...	92	1e-18
sp|P57282|PPNK_BUCAI PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	92	1e-18
sp|Q9HKH7|PPNK_THEAC PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	87	5e-17
sp|P58058|PPNK_MOUSE PUTATIVE INORGANIC POLYPHOSPHATE/ATP-NAD K...	87	8e-17
sp|Q9HNX7|PPNK_HALN1 PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	85	3e-16
sp|O30297|PPNK_ARCFU PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	84	5e-16
sp|O95544|PPNK_HUMAN PUTATIVE INORGANIC POLYPHOSPHATE/ATP-NAD K...	83	7e-16
sp|O25944|PPNK_HELPY PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	83	7e-16
sp|P32622|YEF1_YEAST HYPOTHETICAL 55.9 KDA PROTEIN IN GDA1-UTR2...	82	3e-15
sp|Q9ZJ81|PPNK_HELPJ PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	81	4e-15
sp|Q9X255|PPNK_THEMA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	81	4e-15
sp|Q06892|POS5_YEAST POS5 PROTEIN.	80	6e-15
sp|Q9PHM6|PPNK_CAMJE PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	79	2e-14
sp|P21373|UTR1_YEAST UTR1 PROTEIN (UNKNOWN TRANSCRIPT 1 PROTEIN).	79	2e-14
sp|Q9YD08|PPNK_AERPE PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	77	5e-14
sp|O13863|YDU2_SCHPO HYPOTHETICAL 60.7 KDA PROTEIN C1B1.02C IN ...	76	9e-14
sp|Q9K904|PPNK_BACHD PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	71	4e-12
sp|P58055|PPNK_BACST PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	71	4e-12
sp|Q9CIJ4|PPNK_LACLA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	68	4e-11
sp|O31612|PPNK_BACSU PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	68	5e-11
sp|P58056|PPNK_CAUCR PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	59	2e-08
sp|Q9PBQ0|PPNK_XYLFA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	54	4e-07
sp|Q9ZDA2|PPNK_RICPR PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	53	8e-07
sp|P75508|PPNK_MYCPN PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	46	2e-04
sp|P47374|PPNK_MYCGE PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	41	0.003
sp|Q9PQW6|PPNK_UREPA PROBABLE INORGANIC POLYPHOSPHATE/ATP-NAD K...	37	0.064
sp|P78559|MAPA_HUMAN MICROTUBULE-ASSOCIATED PROTEIN 1A [CONTAIN...	32	1.6
sp|P56900|TKT_RHIME TRANSKETOLASE (EC 2.2.1.1) (TK).	32	2.8
sp|P49376|ATPB_KLULA ATP SYNTHASE BETA CHAIN, MITOCHONDRIAL PRE...	32	2.8
sp|P23895|EMRE_ECOLI EMRE PROTEIN (METHYL VIOLOGEN RESISTANCE P...	31	4.8
sp|P38387|SECD_MYCLE PROTEIN-EXPORT MEMBRANE PROTEIN SECD.	31	6.3
sp|P16920|SECE_ECOLI PREPROTEIN TRANSLOCASE SECE SUBUNIT.	31	6.3
sp|O83384|Y369_TREPA HYPOTHETICAL PROTEIN TP0369 PRECURSOR.	31	6.3
sp|O26361|IF2G_METTH PROBABLE TRANSLATION INITIATION FACTOR 2 G...	31	6.3

TABLE 3 Other features of proteins listed in Table 2

ID	Source	A.A.	M.M. (Da)
|Q9S219|PPNK_STRCO	*Streptomyces coelicolor*	301	32081
|O33196|PPNK_MYCTU	*Mycobacterium tuberculosis*	307	32903
|Q49897|PPNK_MYCLE	*Mycobacterium leprae*	311	33218
|Q9KTP8|PPNK_VIBCH	*Vibrio cholerae*	294	32698
|Q9HZC0|PPNK_PSEAE	*Pseudomonas aeruginosa*	295	32140
|O26958|PPNK_METTH	*Methanobacterium thermoautotrophicum*	283	31603
|Q9CNU2|PPNK_PASMU	*Pasteurella multocida*	305	33710
|Q51841|PPNK_PORGI	*Porphyromonas gingivalis* (*Bacteroides gingivalis*)	288	31805
|O83455|PPNK_TREPA	*Treponema pallidum*	305	32633
|O67055|PPNK_AQUAE	*Aquifex aeolicus*	274	31327
|P44497|PPNK_HAEIN	*Haemophilus influenzae*	285	31828
|Q58327|PPNK_METJA	*Methanococcus jannaschii*	574	64118
|O51291|PPNK_BORBU	*Borrelia burgdorferi* (*Lyme disease spirochete*)	279	30966
|P74430|PPN1_SYNY3	*Synechocystis* sp. (strain PCC 6803)	305	33397
|Q9JQL9|PPNK_NEIMA	*Neisseria meningitidis* (serogroup A), and *Neisseria meningitidis* (serogroup B)	296	32852
|P73955|PPN2_SYNY3	*Synechocystis* sp. (strain PCC 6803)	307	33775
|P37768|PPNK_ECOLI	*Escherichia coli*	292	32566
|P58057|PPNK_ECO57	*Escherichia coli* O157:H7	292	32596
|O58801|PPNK_PYRHO	*Pyrococcus horikoshii*	277	31414
|Q9V081|PPNK_PYRAB	*Pyrococcus abyssi*	277	31672
|P57282|PPNK_BUCAI	*Buchnera aphidicola* (subsp. *Acyrthosiphon pisum*) (*Acyrthosiphon pisum symbiotic bacterium*)	292	32576
|Q9HKH7|PPNK_THEAC	*Thermoplasma acidophilum*	272	30087
|P58058|PPNK_MOUSE	*Mus musculus* (Mouse)	439	48625
|Q9HNX7|PPNK_HALN1	*Halobacterium* sp. (strain NRC-1)	282	29120
|O30297|PPNK_ARCFU	*Archaeoglobus fulgidus*	249	27868
|O95544|PPNK_HUMAN	*Homo sapiens* (Human)	446	49228
|O25944|PPNK_HELPY	*Helicobacter pylori* (*Campylobacter pylori*)	284	31631
|P32622|YEF1_YEAST	*Saccharomyces cerevisiae* (Baker's yeast)	495	55873
|Q9ZJ81|PPNK_HELPJ	*Helicobacter pylori* J99 (*Campylobacter pylori* J99)	284	31447
|Q9X255|PPNK_THEMA	*Thermotoga maritima*	258	29241
|Q06892|POS5_YEAST	*Saccharomyces cerevisiae* (Baker's yeast)	414	46246
|Q9PHM6|PPNK_CAMJE	*Campylobacter jejuni*	286	32381
|P21373|UTR1_YEAST	*Saccharomyces cerevisiae* (Baker's yeast)	530	59469
|Q9YD08|PPNK_AERPE	*Aeropyrum pernix*	291	32225
|O13863|YDU2_SCHPO	*Schizosaccharomyces pombe* (Fission yeast)	537	60708
|Q9K904|PPNK_BACHD	*Bacillus halodurans*	265	29872
|P58055|PPNK_BACST	*Bacillus stearothermophilus*	271	30517
|Q9CIJ4|PPNK_LACLA	*Lactococcus lactis* (subsp. *lactis*) (*Streptococcus lactis*)	270	30381
|O31612|PPNK_BACSU	*Bacillus subtilis*	266	30012
|P58056|PPNK_CAUCR	*Caulobacter crescentus*	260	28524
|Q9PBQ0|PPNK_XYLFA	*Xylella fastidiosa*	259	28852
|Q9ZDA2|PPNK_RICPR	*Rickettsia prowazekii*	255	28775
|P75508|PPNK_MYCPN	*Mycoplasma pneumoniae*	259	29033
|P47374|PPNK_MYCGE	*Mycoplasma genitalium*	259	29199
|Q9PQW6|PPNK_UREPA	*Ureaplasma parvum* (*Ureaplasma urealyticum* biotype 1)	270	31358
|P78559|MAPA_HUMAN	*Homo sapiens* (Human)	2805	306051
|P56900|TKT_RHIME	*Rhizobium meliloti* (*Sinorhizobium meliloti*)	695	75693
|P49376|ATPB_KLULA	*Kluyveromyces lactis* (Yeast)	505	54068
|P23895|EMRE_ECOLI	*Escherichia coli*	110	11958
|P38387|SECD_MYCLE	*Mycobacterium leprae*	571	60718
|P16920|SECE_ECOLI	*Escherichia coli*	127	13643
|O83384|Y369_TREPA	*Treponema pallidum*	516	55859

A.A., Numbers of amino acid residues. M.M., Molecular mass.

```
Mfnk            MPYTPGRRILVLTHTGREDAISAALQATRMFAEEGLVTVMLEQDVAAIRAAAGDPPEF--
sp>Q9S219-1     ------RTVFLLAHTGRPAAIRSAELVVKGLLRAGIGVRVLE-------AEARDLPLP--
sp>O33196-1     ---TAHRSVLLVVHTGRDEATETARRVEKVLGDNKIALRVLSAEAVDRGSLHLAPDDM--
sp>Q49897-1     ------RTVLLVVHTGRDEATETARRVKKIVGDNGIALRVLSAEAVDRGSLHLALDNM--
sp>Q9KTP8-1     ------------------------------------------------------------
sp>Q9HZC0-1     ----PFRNIGIIGRLGSTQVLDT-IRRLKKFLIDRHLHVILED--------TIAEVLP--
sp>O26958-1     ------------------------------------------------------------
sp>Q9CNU2-1     ------------------------------------------------------------
sp>Q51841-1     ------------------------------------------------------------
sp>O83455-1     ------------------------------------------------------------
sp>O67055-1     ------------------------------------------------------------
sp>P44497-1     ------------------------------------------------------------
sp>Q58327-1     ------------------------------------------------------------
sp>O51291-1     ------------------------------------------------------------
sp>P74430-1     ------------------------------------------------------------
sp>Q9JQL9-1     ------------------------------------------------------------
sp>P73955-1     ------------------------------------------------------------
sp>P37768-1     ------------------------------------------------------------
sp>P58057-1     ------------------------------------------------------------
sp>O58801-1     ------------------------------------------------------------
sp>Q9V081-1     ------------------------------------------------------------
sp>P57282-1     ------------------------------------------------------------
sp>Q9HKH7-1     ------------------------------------------------------------
sp>P58058-1     ------------------------------------------------------------
sp>Q9HNX7-1     ------------------------------------------------------------
sp>O30297-1     ------------------------------------------------------------
sp>O95544-1     ------------------------------------------------------------
sp>O25944-1     ------------------------------------------------------------
sp>P32622-1     ------------------------------------------------------------
sp>Q9ZJ81-1     ------------------------------------------------------------
sp>Q9X255-1     ------------------------------------------------------------
sp>Q06892-1     ------------------------------------------------------------
sp>Q9PHM6-1     ------------------------------------------------------------
sp>P21373-1     ------------------------------------------------------------
sp>Q9YD08-1     ------------------------------------------------------------
sp>O13863-1     ------------------------------------------------------------
sp>Q9K904-1     ------------------------------------------------------------
sp>P58055-1     ------------------------------------------------------------
sp>Q9CIJ4-1     ------------------------------------------------------------
sp>O31612-1     ------------------------------------------------------------
sp>P58056-1     ------------------------------------------------------------
sp>Q9PBQ0-1     ------------------------------------------------------------A
sp>Q9ZDA2-1     ------------------------------------------------------------
sp>P75508-1     ------------------------------------------------------------
sp>P47374-1     ------------------------------------------------------------
sp>Q9PQW6-1     ------------------------------------------------------------
```

FIG. 2-1 Alignment of primary structures of proteins showing homology with that of Mfnk.

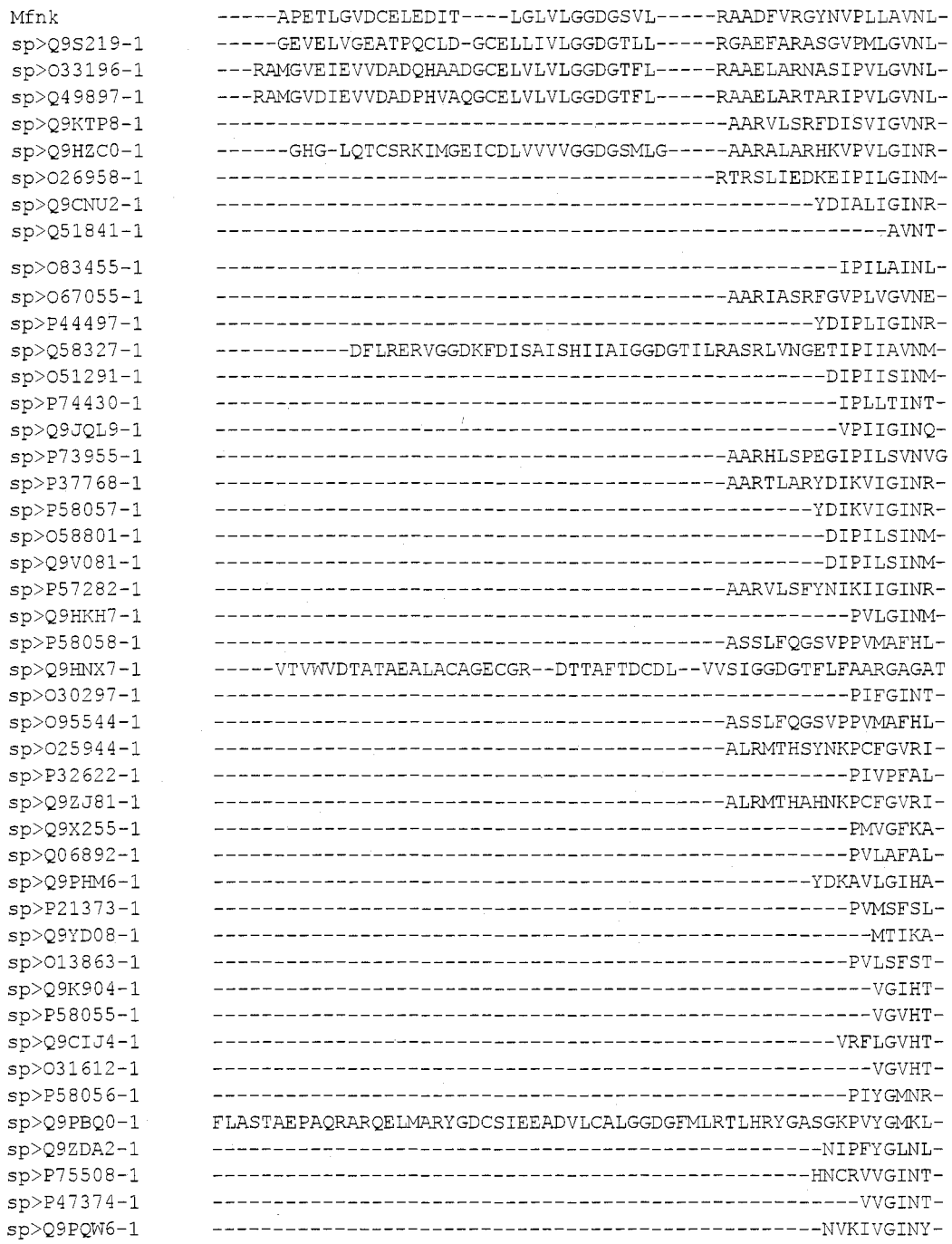

FIG. 2-2 Alignment of primary structures of proteins showing homology with that of Mfnk.

```
Mfnk            ----------GHVGFLAESE-------RTDLHRTVQAIASE-SYVVIERMALD----V
sp>Q9S219-1     ----------GRVGFLAEAE-------RDDLDKVVDRVVNR-AYEVEERMTVD----V
sp>O33196-1     ----------GRIGFLAEAE-------AEAIDAVLEHVVAQ-DYRVEDRLTLD----V
sp>Q49897-1     ----------GRIGFLAEAE-------AEAIDVVLEHVIAR-SYRVEERLTLD----I
sp>Q9KTP8-1     ----------GNLGFLTDLN-------PEDFQQRLQEVLDG-HYLQETRFLLE----A
sp>Q9HZC0-1     ----------GSLGFLTDIR-------PDELEAKVGEVLDG-QYIVESRFLLD----A
sp>O26958-1     ----------GTVGFLTEVD-------PENVFSALEAVLRG-EYAVEKRTLLS----V
sp>Q9CNU2-1     ----------GNLGFLTDID-------PKNAYSQLQACLEDGDCFVEERFILE----A
sp>Q51841-1     ----------GRLGFLTDVD-------CHEASELITRLLDG-DFTIETRSLLEVT---
sp>O83455-1     ----------GRFGFIAPIEP------RYWQQALSDYLAG-GVRPAERALIS----C
sp>O67055-1     ----------GRFGFLTEIK-------KEEIKKVLPLVLEG-RAKLQERLMID-----
sp>P44497-1     ----------GNLGFLTDID-------PKNAYSQLEACLERGEFFVEERFLLE----A
sp>Q58327-1     ----------GKVGFLAEFC-------KDEVFEIIDKVIYG-EYEIEKRSKLS----C
sp>O51291-1     ----------GNVGFLADIK-------IEDFKKVIDRFFNN-SLVINKKFLLH----V
sp>P74430-1     ----------GHMGFLTEIY-------LNQLPTAIEQLING-DYQIESRSMMT-----
sp>Q9JQL9-1     ----------GHLGFLTQIP-------REYMTDKLLPVLEG-KYLAEERILIE----A
sp>P73955-1     ----------GHLGFLTEPFD------VFQDTQKVWDRLNQDRYAVSQRMMLAASLFE
sp>P37768-1     ----------GNLGFLTDLD-------PDNAQQQLADVLEG-HYISEKRFLLE----A
sp>P58057-1     ----------GNLGFLTDLD-------PDNAQQQLADVLEG-HYISEKRFLLE----A
sp>O58801-1     ----------GTLGFLTEVE-------PSDTFFALNRLIEG-EYYIDERIKVR----T
sp>Q9V081-1     ----------GTLGFLTEVE-------PSETFFAINRLLRG-EYYIDERIKLR----T
sp>P57282-1     ----------GNLGFLADLN-------PDTGLKKLSEVLSG-NYSLENRFLLD----A
sp>Q9HKH7-1     ----------GGLGFLTELE-------VDEVGSAIFKLIKG-QYRITESMKLKVE-IN
sp>P58058-1     ----------GSLGFLTPFN-------FENFQSQVNQVIEG-NAAVILRSRLK----V
sp>Q9HNX7-1     -----PILGVNLGEVGFLNAVAP-------ADAVEAVREEVNRYRETGAVRCREVP----
sp>O30297-1     ----------GRVGLLT-------HASPENFEVELKKAVEKFEVERFPRVSCS-----
sp>O95544-1     ----------GSLGFLTPFS-------FENFQSQVTQVIEG-NAAVVLRSRLK----V
sp>O25944-1     ----------GNLGFLSAVE-------LNGLKDFLQDFKQD-RIKLEEHLAL------
sp>P32622-1     ----------GSLGFLTNFE-------FQNFKETLKHILTD-EVRINLRMRLQ----C
sp>Q9ZJ81-1     ----------GNLGFLSAVE-------LNGLKDFLQDLKQN-RIKLEEHLAL------
sp>Q9X255-1     ----------GRLGFLTSYT--------LDEIDRFLEDLRNWNFREETRWFIQIE---
sp>Q06892-1     ----------GTLGFLSPFD-------FKEHKKVFQEVISS-RAKCLHRTRLE----C
sp>Q9PHM6-1     ----------GHLGFLTDFK-------VDEAENFFQAFFQG-EFRIEKPYLLS-----
sp>P21373-1     ----------GSLGFLTNFK-------FEHFREDLPRIMNH-KIKTNLRLRLE----C
sp>Q9YD08-1     ----------GKKGFLLDVER-------YEAVERLRDFLEG-RFREVVYPRYR-----
sp>O13863-1     ----------AKAGFLSILP-------IAEYTKTLDLIFHR-GFTVNLRMRFQ----C
sp>Q9K904-1     ----------GHLGFYADWV-------PDEVEKLVIHIAKT-PYQIVEYPL------L
sp>P58055-1     ----------GHLGFYADWV-------PEELEKLVIAIAKT-PYQVVEYPL------L
sp>Q9CIJ4-1     ----------GHLGFYTDFT-------DEDLFEVVEALYDENPAQAIHYPL------I
sp>O31612-1     ----------GHLGFYADWV-------PHEIEKLVLAIAKT-PYHTVEYPL------L
sp>P58056-1     ----------GSVGFLMNEY-------SED--GLLERINAAERAVIHPLAM------V
sp>Q9PBQ0-1     ----------GSVGFLMNQY-------HDD---LLERLQRAEPAKLRPLQMMA-----
sp>Q9ZDA2-1     ----------GSLGFLMNP---------LDTKKLLQNIYESTVSILHPLLMQV-----
sp>P75508-1     ----------GHLGFYTSFN-------EKDLDDNFLQKLQQCH--FQRISL------L
sp>P47374-1     ----------GHIGFYTSFN-------GDDLDENFISKLTSFE--FKKINL------L
sp>Q9PQW6-1     ----------GQLGFYSSYD--------SIKTINLDEIIDENMYY--NPLLLKVSINN
                          .  *:              .
```

FIG. 2-3 Alignment of primary structures of proteins showing homology with that of Mfnk.

```
Mfnk            VVHVEGRE-------------------------VARTWALNEASVEKSHRERMLEVVVS
sp>Q9S219-1     VVHRNGDI-------------------------VHTDWALNEAAVQKAGAEKLLEVVLE
sp>O33196-1     VVRQGGRI-------------------------VNRGWALNEVSLEKGPRLGVLGVVVE
sp>Q49897-1     VVRQGGNI-------------------------IDQGWALNEASLEKGPRLGVLGVVVE
sp>Q9KTP8-1     EIHRHGQVK------------------------SHNAALNEAVLHPGKIAHMIEFEVY
sp>Q9HZC0-1     QVRRGIDSM------------------------GQGDALNDVVLHPGKSTRMIEFELY
sp>O26958-1     YHNDELPS-------------------------------ALNEVVLMTRRPAKMLHIEIS
sp>Q9CNU2-1     SVERNGKII------------------------ARGNAVNEAVVHPAKIAHMIDFHVY
sp>Q51841-1     --EDNGSSPSY----------------------------ALNEAAILKRETGSMIRVNAC
sp>O83455-1     TVTRAGKEIAS----------------------CLALNDVVLSSGRVARLTRAEVC
sp>O67055-1     -VYLRSRNRLR----------------------YLGNYLNDAVISKSSIARIIRTKVF
sp>P44497-1     KIERASEIV------------------------STSNAVNEAVIHPAKIAHMIDFHVY
sp>Q58327-1     KIIKDNRVI------------------------KTPSALNEMVVITKNPAKILEFDVY
sp>O51291-1     TVSQHGKDLIS----------------------KYALNDIIIRSSVLNKMIYVDLM
sp>P74430-1     --VRLMREEN----------------------LLWEALSLNEMVLHREPLTSMCHFEIQ
sp>Q9JQL9-1     ALIREGKTA------------------------ERAIALNDAVLSRGGAGQMIEFEVF
sp>P73955-1     GDRRDPQMVG----------------------ETYYCLNEMCIKPASIDRMPTAIIE
sp>P37768-1     QVCQQDCQK------------------------RISTAINEVVLHPGKVAHMIEFEVY
sp>P58057-1     QVCQQDCQK------------------------RISTAINEVVLHPGKVAHMIEFEVY
sp>O58801-1     YIDGENRVP------------------------D---ALNEVAILTGIPGKIIHMKYY
sp>Q9V081-1     YINGEARIP------------------------D---ALNEVAILTGIPGKVIHLRYY
sp>P57282-1     QVCQKKIIS------------------------RSSIAINEVVLHTKNLAHMIEFEVY
sp>Q9HKH7-1     GDRVEDCT-----------------------------NEAVVHTERIARIRQFKIY
sp>P58058-1     RVVKEPRDKKTAIHNGLSENG-----LDTEGGKQAMQYQVLNEVVIDRGPSSYLSNVDVY
sp>Q9HNX7-1     RVVAAGDGW------------------------ASTPALNEVAIQGEQRGHGHGVAVD
sp>O30297-1     ---------------------------------AMPDVLALNEIAVLSRKPAKMIDVALR
sp>O95544-1     RVVKELRGKKTAVHNGLGENGSQAAGLDMDVGKQAMQYQVLNEVVIDRGPSSYLSNVDVY
sp>O25944-1     ----EGR------------------------IGKTSFYAINEIVIAKKKALGVLDIKAY
sp>P32622-1     KLYRRNKPE-------IDAATGR----KICYIDFISEHHVLNEVTIDRGPAPCLSLLELY
sp>Q9ZJ81-1     ----EGR------------------------IGNTSFYAINEIVIAKKKALGVLDIKAC
sp>Q9X255-1     --------------------------------SELGNHLALNDVTLERDLSGKMVEIEVE
sp>Q06892-1     --HLKKKDSNS----------------------SIVTHAMNDIFLHRGNSPHLTNLDIF
sp>Q9PHM6-1     -IFLEDRQGK-----------------------ILEKLAFNDVVISKNNQASMAHIEVF
sp>P21373-1     TIYRRHRPE-------VDPNTGK----KICVVEKLSTHHILNEVTIDRGPSPFLSMLELY
sp>Q9YD08-1     -VYLEGE--------------------------ARACMFNDTAVT-ANNAKMARVHVF
sp>O13863-1     SIMRYVG-----------EHS---------THICEGQYSVLNELLIDRGPNPFMISLDLY
sp>Q9K904-1     EVVVRHTDESE----------------------SKRLL-ALNECTVKS--QEGSLVSNVE
sp>P58055-1     EVTIRYLNGGS----------------------EAKYL-ALNECTVKCVS--GTLVMDVE
sp>Q9CIJ4-1     RVQVSFTDGYQ----------------------IVRH--VLNEATIRRAS--KTMVGDVR
sp>O31612-1     EVIVTYHENER----------------------EERYL-ALNECTIKSI--EGSLVADVE
sp>P58056-1     AIDARRTQHR-----------------------A--LAINEVSLLRQTRQTA-KLRIS
sp>Q9PBQ0-1     -QTESGVSVE-----------------------S--LAYNEVSLLRQTHQAAYISIDL
sp>Q9ZDA2-1     -EDTSGQIYK-----------------------A--LAINEVSIFRKTNQVAKFRIDV
sp>P75508-1     EVSVNGQQHL-----------------------------VLNELAV-----YTNTAYPIN
sp>P47374-1     EVKTKNH--------------------------SFLVLNELAV-----YTNTAYPIN
sp>Q9PQW6-1     QNF-----------------------------------FYCLNELSLFSNE---LVSFDIS
                                                      *:  :
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FIG. 2-4 Alignment of primary structures of proteins showing homology with that of Mfnk.

```
Mfnk            V--DNSPLTSFGCDGVVLATPTGSTAYAFSAGGPVVWPSVEALLCVPISAHALFTR----
sp>Q9S219-1     I--DGRPVTGFGCDGIVLSTPTGSTAYAFSAGGPVVWPEVEALLMVPISAHALFAK----
sp>O33196-1     I--DGRPVSAFGCDGVLVSTPTGSTAYAFSAGGPVLWPDLEAILVVPNNAHALFGR----
sp>Q49897-1     I--EGRPVSTFGCDGVLVSTPTGSTAYAFSAGGPVLWPDLEAILVVPNNAHALFGR----
sp>Q9KTP8-1     I--DDNFAFSQRSDGLIVSTPTGSTAYSLSGGGPILSPSLNAITLVPMFPHTLSCR----
sp>Q9HZC0-1     I--DGQFVCSQKADGLIVATPTGSTAYALSAGGPIMHPKLDAIVIVPMYPHMLSSR----
sp>O26958-1     V--DDEVVEELRADGIIIATPSGSTAYSMSAGGPIVDPRVEAFLIVPICPFKLSAR----
sp>Q9CNU2-1     I--NDKFAFSQRSDGLIISTPTGSTAYSLSAGGPILTPQLNAIALVPMFPHTLSSR----
sp>Q51841-1     LN--DDYLAAYDADGLVVATPSGSTAYSLSGNGPIIMPACRNFVLTPIAPHSLNMR----
sp>O83455-1     FN--DISFGVYEADGIILATPTGSTAYSAACGGPILDPDLDAFVLTPISALCLSNR----
sp>O67055-1     IN--GEEVLEVFGDGVILSTPTGSTAYALSAGGPIVYPESQNLLFVPICPHTLSNR----
sp>P44497-1     I--NDKFAFSQRSDGLIVSTPTGSTAYSLSAGGPILTPNLNAIALVPMFPHTLTSR----
sp>Q58327-1     V--NDTLVENVRADGIIVSTPTGSTAYSLSAGGPIVEPNVDCFIISPICPFKLSSR----
sp>O51291-1     VN--SESFLSYKSDGIIVSTPTGSTGYSFSAGGPILEADLEGFXLTPISPHSVYNR----
sp>P74430-1     VG--YHASVDIAADGIIVSTPTGSTAYSLSAGGPVVTPDVPVFQLAPICPHSLASR----
sp>Q9JQL9-1     V--NREFVYTQRSDGLIVSTPTGSTAYSLAAGGPIMQAGLHAFTLVPICPQSMTNR----
sp>P73955-1     VEVDGELIDQYQCDGLLVATPTGSTCYTSSANGPILHPGMDAIVITPICPLSLSSR----
sp>P37768-1     I--DEIFAFSQRSDGLIISTPTGSTAYSLSAGGPILTPSLDAITLVPMFPHTLSAR----
sp>P58057-1     I--DEIFAFSQRSDGLIISTPTGSTAYSLSAGGPILTPSLDAITLVPMFPHTLSAR----
sp>O58801-1     V--DGGLADEVRADGLVVSTPTGSTGYAMSAGGPFIDPRLDVILIAPLLPLPKTSV----
sp>Q9V081-1     V--DGGLADEVRADGLVVATPTGSTGYAMSAGGPFVDPRLDTIIIAPLLPLPRTSV----
sp>P57282-1     I--DNKFSFSQRADGLIVSTPTGSTGYSLSAGGPIIAASLDAIVLVPMFPHTLSAR----
sp>Q9HKH7-1     I--DGHFLSTMKSDGIIVATPIGSSSYSSSAGGPLLLPTLKGMVISYLAPYSSRLK----
sp>P58058-1     L--DGHLITTVQGDGVIVSTPTGSTAYAAAAGASMVHPNVPAIMVTPICPHSLSFR----
sp>Q9HNX7-1     VRVDGSQYEATRADGVLVATPTGSTAYNLSEGGPLVQPSVDALVVTEMCGADAL------
sp>O30297-1     V--DGVEVDRIRCDGFIVATQIGSTGYAFSAGGPVVEPYLECFILIPIAPFRFGWK----
sp>O95544-1     L--DGHLITTVQGDGVIVSTPTGSTAYAAAAGASMIHPNVPAIMITPICPHSLSFR----
sp>O25944-1     A--GHTPFNTYKGDGLIIATPLGSTAYNLSAHGPIVHALSQSYILTPLCDFSLTQR----
sp>P32622-1     G--NDSLMTKVQGDGLIVATPTGSTAYSLSAGGSLISPSVNAIAVTPICPHTLSFR----
sp>Q9ZJ81-1     A--GHTPFNTYKGDGLIIATPLGSTAYNLSAHGPIVHALSQSYILTPLCDFSLTQR----
sp>Q9X255-1     V--EHHSSMWFFADGVVISTPTGSTAYSLSIGGPIIFPECEVLEISPIAPQFFLTR----
sp>Q06892-1     I--DGEFLTRTTADGVALATPTGSTAYSLSAGGSIVSPLVPAILMTPICPRSLSFR----
sp>Q9PHM6-1     R--KEKKFNEYFGDGLIVATPAGSTAYNLSANGPIVYTLAQAFILTPVCSHSLTQR----
sp>P21373-1     G--DGSLMTVAQADGLIAATPTGSTAYSLSAGGSLVCPTVNAIALTPICPHALSFR----
sp>Q9YD08-1     V--DGDLAMNIDGDGVVVSTTAGSTAYSLSGGGPIIDPRLDVIVLTPLNPVQLFLR----
sp>O13863-1     V--ENEYITTLQSDGVCVSTPTGSTAYSVAAGGSLCHPGIPAILISAICPHSLSFR----
sp>Q9K904-1     IKGDV--FEVFRGDGLCISTPSGSTAYNKALGGAILHPSLASIQISEMASINNRVYR---
sp>P58055-1     IRGDL--FERFRGDGLCISTPTGSTAYNKALGGAILHPSLEAIQVTEMASINNRVF----
sp>Q9CIJ4-1     IS--DYLFERFRGDGLSISTPTGSTAYNKSIGGAVVHPRVKAMQVAEIASLNNVVYR---
sp>O31612-1     IKGQL--FETFRGDGLCLSTPSGSTAYNKALGGAIIHPSIRAIQLAEMASINNRVFR---
sp>P58056-1     ID-GKVRMGELVCDGALLATPAGSTAYNLSAHGPII----------PIDGRVLALT----
sp>Q9PBQ0-1     N--GQTRIDELTGDGVIVATPAGSTAYNYSAHGPIL----------PLGSHTLALT----
sp>Q9ZDA2-1     N--GVERMSELVADGALVATPAGSSAYNLSAGGPIL----------PLASNMLCL-----
sp>P75508-1     IFIDGEAWEFYRGSGLLIGPRTGSTALAKSAKGAVIFPGIDVLQIIEMNPLLHPNQV---
sp>P47374-1     IFIDDNHWESYRGSGLLIGPRTGSTALAKSAKGAVIFPNVDVVQIIELN-----------
sp>Q9PQW6-1     IN--DYPYEKFRGSGLLFVTPSGSTGKNKTAFGPIIFNNHENFIMTEI-----------
                              .*    .  **:    :  ...
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FIG. 2-5 Alignment of primary structures of proteins showing homology with that of Mfnk.

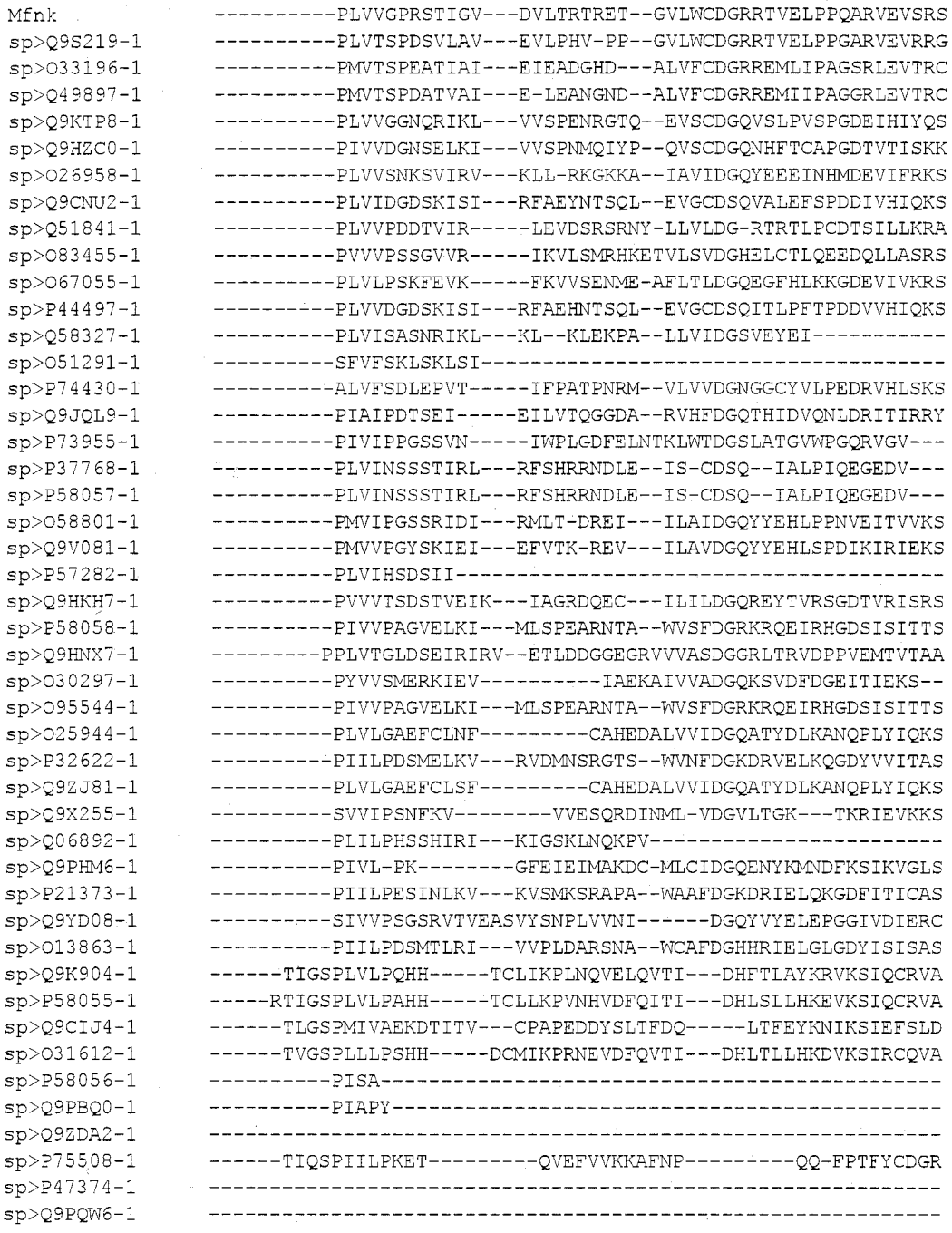

FIG. 2-6 Alignment of primary structures of proteins showing homology with that of Mfnk.

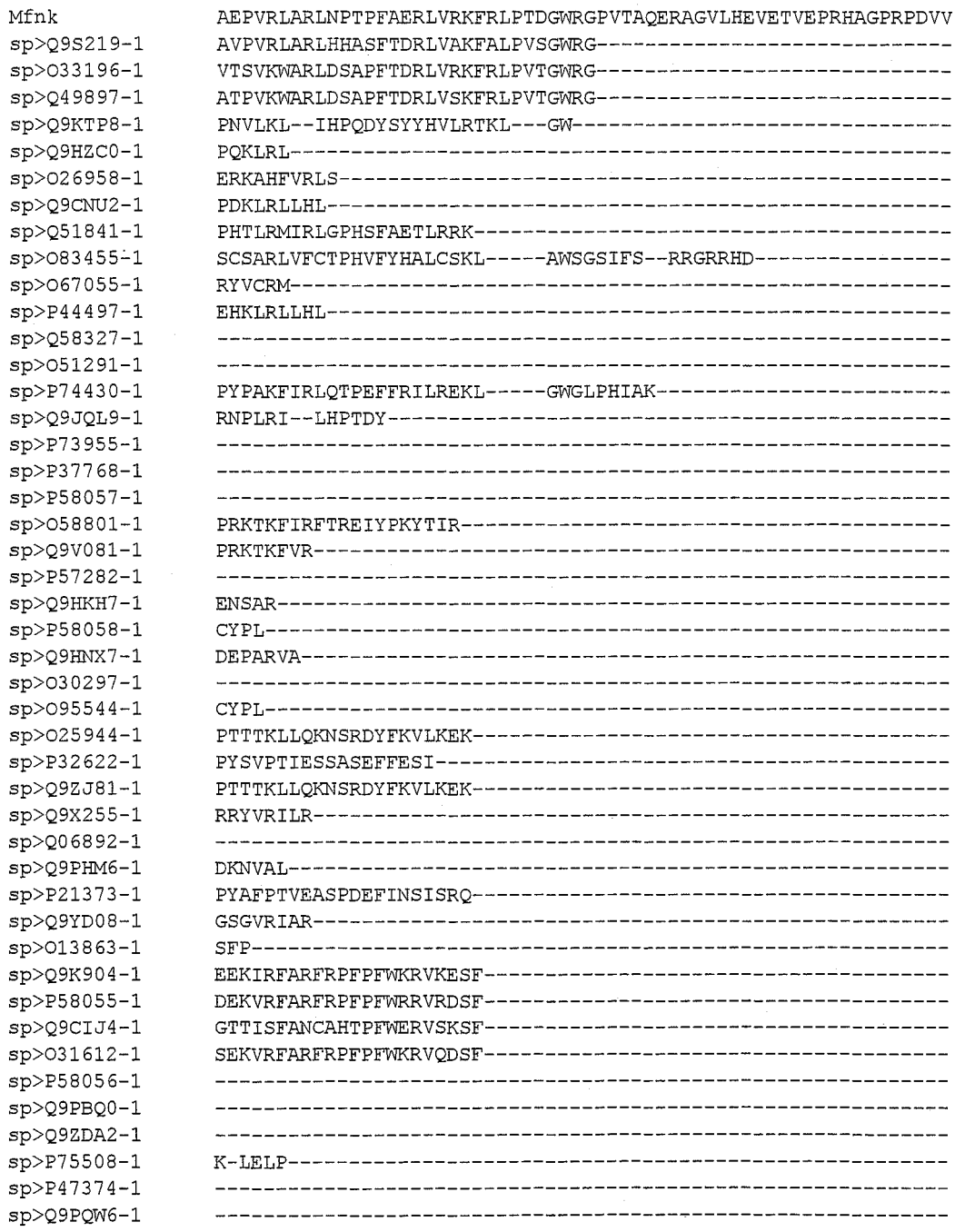

FIG. 2-7 Alignment of primary structures of proteins showing homology with that of Mfnk.

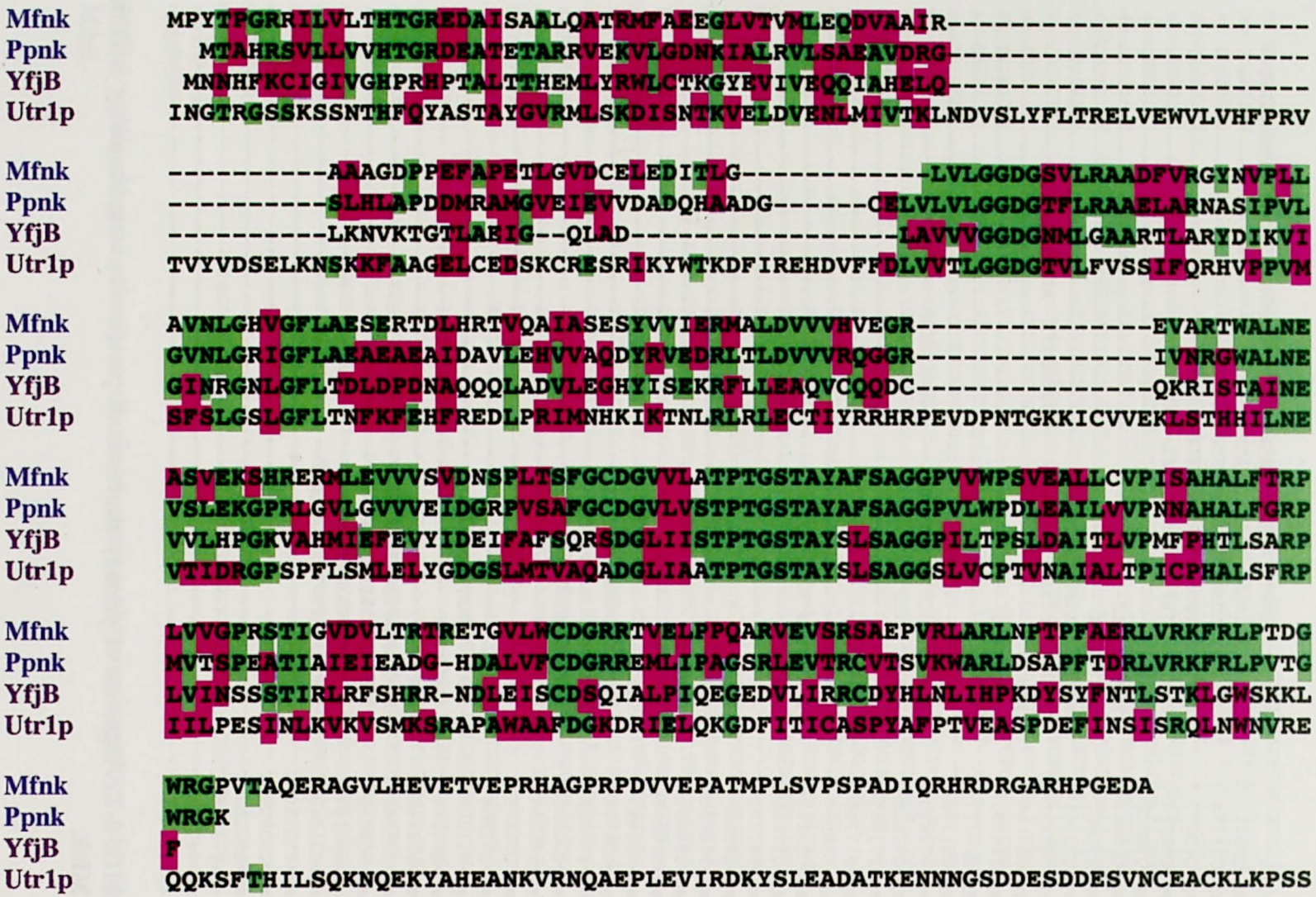

FIG. 3 Alignment of primary structures of Mfnk, Ppnk, YfjB, and Utr1p. Identical and similar amino acid residues with Mfnk and Ppnk are colored with green and purple, respectively.

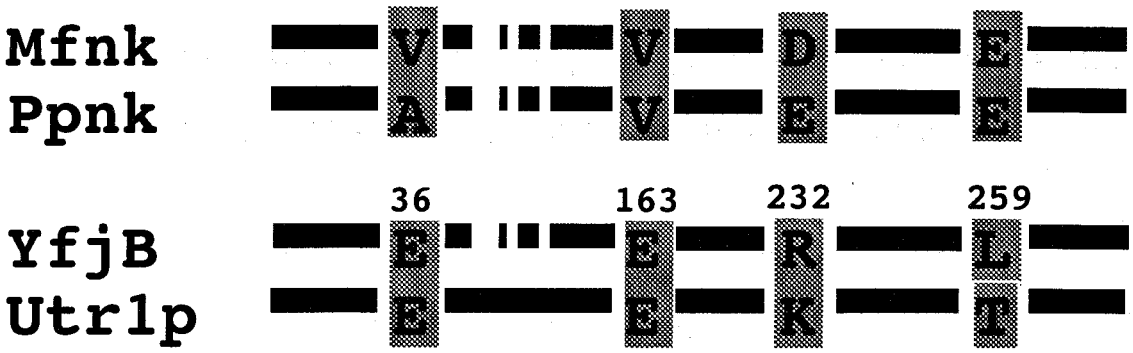

FIG. 4 Alignment of primary structures of Mfnk, Ppnk, YfjB, and Utr1p. Characteristic amino acid residues of poly(P)/ATP- and ATP-NAD kinases are boxed. Amino acid residues of YfjB are numbered.

TABLE 4 Poly(P)- and ATP-dependent NAD kinase activities of YfjB mutants

Mutants	Poly(P)-dependent activity (units/mg)	ATP-dependent activity (units/mg)	[(a)/(b)]x100 (%)
None	0.096	3.92	2.4
E36A	0.095	3.16	3.0
E163V	0.023	0.77	3.0
R232D	0.027	0.93	2.9
L259E	0.074	2.64	2.8
E36A/E163V	0.010	0.35	2.7
R232D/L259E	0.018	0.62	2.9
E36A/E163V/ R232D/L259E	0.001	0.04	2.8

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Section 4 Crystallization and preliminary X-ray analysis of inorganic polyphosphate/ATP-NAD kinase

As described in the previous Section, in spite of the significant difference of phosphoryl donor and acceptor specificities of poly(P)/ATP-NAD kinases (Mfnk and Ppnk) and ATP-NAD kinases (YfjB and Utr1p), primary structures of the enzymes are highly conserved. This conservation indicates that molecular evolution of NAD kinase, acquirement or loss of the ability to utilize NADH and especially poly(P) in an evolutionary process, should occur as a result of accumulation of point mutations with limited numbers.

In order to understand the evolutionary process from a standpoint of structural biology, or in other words, to identify amino acid residues for poly(P) utilization, determination of three dimensional structures of poly(P)/ATP- and ATP-NAD kinases and comparison of them are indispensable. In this Section, crystallization and preliminary X-ray analysis of poly(P)/ATP-NAD kinase (Ppnk) of *Mycobacterium tuberculosis* H37Rv are described.

MATERIALS AND METHODS

Purification of poly(P)/ATP-NAD kinase (Ppnk). Poly(P)/ATP-NAD kinase (Ppnk) of *M. tuberculosis* H37Rv was purified as described in Section 1 of this Chapter. The purified Ppnk was dialyzed against 10 mM potassium phosphate (pH 7.0) containing 0.20 mM NAD, 1.0 mM EDTA, and 0.50 mM dithiothreitol, and the dialysate was concentrated by ultrafiltration with a Centrisalts (Saltrius, Tokyo, Japan) to give a final concentration of 6 mg/ml that was determined by the method of Bradford (1).

Crystallization of Ppnk. Crystallization of Ppnk was achieved by the hanging-drop vapour-diffusion method on Linbro tissue-culture plates. A hanging-drop (6 μ l) was prepared by mixing 3 μ l each of Ppnk and the reservoir solution containing 1.8 % polyethylene glycol 4,000, and 100 mM sodium 2-morpholinoethanesulfonic acid (pH6.0), on a siliconized coverslip and kept over 0.50 ml of the reservoir solution.

X-ray analysis. A crystal was mounted in a thin-walled glass capillary for X-ray analysis. Both ends of the capillary were filled with the reservoir solution and then sealed with wax. The diffraction data for a native crystal up to 2.99 Å were collected with a Bruker Hi-Star multiwire area detector at 293 K, using Cu $K\alpha$ radiation generated by a MAC Science

M18XHF rotating anode generator, and were processed with the *SADIE* and *SAINT* software packages (Bruker).

RESULTS AND DISCUSSION

Preliminary X-ray analysis. Prismatic, colorless crystal of Ppnk formed in about 7 days at 293 K and the crystal grew to a maximum size of 0.50 mm (Fig. 1). Preliminary characterization of the crystal indicated monoclinic space group *C*2 with unit-cell parameters of a = 140.01, b = 69.3, c = 106.3 Å, and β = 130.1°. Among all the reflections (33,857) observed, 12,763 independent reflections were obtained with an R_{sym} value of 4.3 %. The data exhibited completeness of 98.9 % up to 2.99 Å resolution. Data collection statistics for the crystal are summarized in Table 1. Two subunits of Ppnk per asymmetric unit give V_M value of 3.28 Å Da^{-1} and a solvent content of 62%. The V_M value and solvent content lie within the range usually found for protein crystals (2).

A search for heavy atom derivatives for phasing with the multiple isomorphous replacement method is now in progress.

TABLE 1 Data collection statistics for NAD kinase crystal

X-ray source	Cu $K\alpha$
Wavelength (Å)	1.54
Resolution (Å)	25.5 - 2.99 (3.17 – 2.99)
Space group	*C*2
Unit-cell parameters (Å)	a=140.0, b=69.3, c=106.3
	$\alpha = \gamma$ =90° β =130.01°
Total observations	42,821
Independent reflections	15,802 (2,527)
Completeness (%)	98.9 (96.6)
I /sigma	2.73 (1.85)
R_{sym} (%)	4.3 (35.0)

Values in parentheses refer to data in the highest resolution shell.

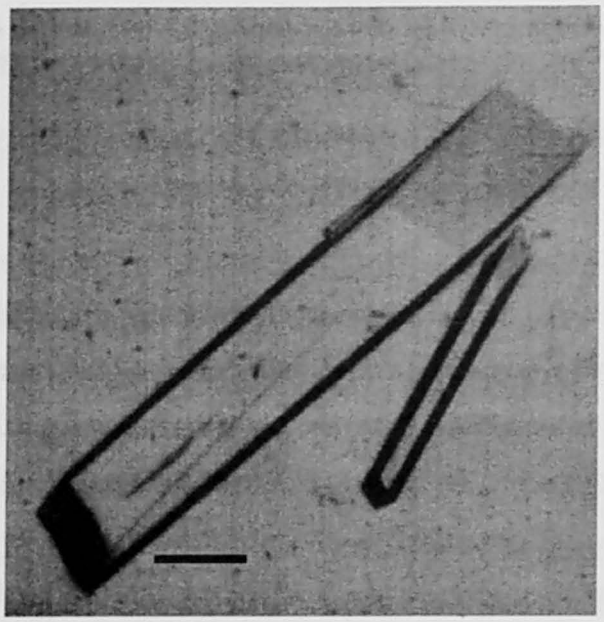

FIG. 1 Crystal of Ppnk. The scale bar is 0.10 mm long.

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Chapter IV

Establishment of Mass-production System of NADP with Inorganic Polyphosphate/ATP-NAD Kinase

NADP is utilized as a diagnostic reagent for enzymatic analysis of blood and urine (1), and is currently produced by means of enzymatic method involving an ATP-NAD kinase (EC 2.7.1.23) that was shown in Chapter II to catalyze the phosphorylation of NAD by the use of ATP, not poly(P) (2):

$$NAD + ATP \rightarrow NADP + ADP$$

However, this production method is not always practical, since ATP is expensive, and the stability as well as the cellular content of the enzyme is low (3). In order to overcome these disadvantages, an attempt was made to use inorganic polyphosphate [poly(P)]-dependent NAD kinase of *Brevibacterium ammoniagenes* to produce NADP from NAD and poly(P) (4, 5). Poly(P) is a polymer of inorganic orthophosphate residues linked through phosphoanhydride bonds energetically equivalent to that of ATP (6), and is commercially obtainable in large quantities at extremely low cost. Although NADP was confirmed to be produced from NAD and poly(P) through the action of poly(P)-dependent NAD kinase, the productivity was far from the practical use (4). This was presumably due to the low activity of poly(P)-dependent NAD kinase in *B. ammoniagenes* cells.

In Section 1 of Chapter III, a gene (*ppnk*) for poly(P)/ATP-NAD kinase (Ppnk) of *Mycobacterium tuberculosis* H37Rv was cloned and expressed in *Escherichia coli*. The enzyme specifically phosphorylated NAD to NADP in the presence of either poly(P) or ATP:

$$NAD + \begin{pmatrix} \text{poly(P)n} \\ \text{ATP} \end{pmatrix} \rightarrow NADP + \begin{pmatrix} \text{poly(P)n-1} \\ \text{ADP} \end{pmatrix}$$

The *E. coli* cells overexpressing *M. tuberculosis* Ppnk showed approximately 400-fold higher poly(P)-dependent NAD kinase activity than that of *B. ammoniagenes* (4, 5). Ppnk purified from recombinant *E. coli* cells or *E. coli* cells immobilized in polyacrylamide gel were successfully utilized for the production of NADP from NAD and poly(P). This NADP production system was thought to be feasible in the production of NADP on an industrial scale. This Chapter deals with the results.

MATERIALS AND METHODS

Strains and growth conditions. Two derivative strains, SK27 and SK45 (chapter I), of *E. coli* BL21(DE3)pLysS (Novagen, Darmstadt, Germany) were used. SK27 carries *ppnk*, the gene for poly(P)/ATP-NAD kinase (Ppnk) of *M. tuberculosis* H37Rv, under the T7 promoter on pET-3a (Novagen). SK45 harbors only pET-3a. In order to overexpress Ppnk, SK27 cells were cultured as described in Chapter I in Luria-Bertani (LB) medium (7) at 18 ℃ for 3 days after induction with 0.40 mM isopropyl-β-D-thiogalactopyranoside. SK45 cells were also treated similarly.

Assay for poly(P)-dependent NAD kinase activity of Ppnk. The poly(P)-dependent NAD kinase activity of Ppnk was assayed as described previously (5) and in Section 1 of Chapter I. One unit of Ppnk activity was defined as 1.0 μmol of NADP produced in 1 h at 37 ℃. Specific activity was expressed in units/mg protein. Protein concentrations were determined by the method of Bradford (8) with bovine serum albumin as a standard.

Purification of Ppnk. Ppnk was purified as described in Chapter I from a cell extract of SK27 overexpressing Ppnk.

Immobilization of cells in polyacrylamide gel. Cells of SK27 or SK45 cultured as above were collected and washed twice with cold 0.85 % NaCl. Immobilization of the cells was performed as described (9) with slight modifications. 5.0 g (wet wt.) of SK27 or SK45 cells was suspended in 6.0 ml of 0.75 M Tris-HCl (pH 8.8). The cell suspension was thoroughly mixed with 4.5 ml of an acrylamide solution (30 % acrylamide, 0.6 % *N*, *N'*-methylenebisacrylamide, 0.25 % *N*, *N*, *N'*, *N'*- tetramethylethylenediamine, and 0.25 % ammonium potassium persulfate), and then kept for 30 min at 0 ℃. The resulting gel (15 ml) was cut into cubes (3.0 mm x 3.0 mm x 3.0 mm), suspended in 50 ml of acetone, and then incubated for 5 min at 0 ℃ with gentle stirring. The gel was washed twice with cold 5.0 mM Tris-HCl (pH 7.0), and then kept in the same buffer supplemented with 0.10 mM NAD and 0.10 mM MgCl2, at 4 ℃ until use. With this method, 5.0 g (wet wt.) of SK27 or SK45 cells was immobilized in 15 ml of polyacrylamide gel, i.e. about 0.33 g (wet wt.) cells/ml-gel.

Preparation of homogenates. Homogenates of intact and acetone-treated cells were prepared by disrupting the cells at 0 ℃ for 10 min in 5.0 ml of 5.0 mM Tris-HCl (pH 7.0) with a Sonifier (Branson, Danbury, CT). Homogenates of immobilized cells and acetone-treated immobilized cells were prepared by grinding 3.0 ml of gel in a pestle with 5.0 ml of

5.0 mM Tris-HCl (pH 7.0) at 0 ℃ for 20 min (10).

Assay for NADP-producing activity. The NADP-producing reaction was carried out at 37 ℃ with shaking in a reaction mixture (4.0 ml) consisting of 50 mM NAD, 100 mM MgCl2, 100 mM Tris-HCl (pH7.0), 50 mg/ml poly(P) (metaphosphate), and purified Ppnk (0.24 units, i.e. 0.16 mg protein), or one of various cell preparations [cells (0.03 g) or immobilized cells (0.10 g), or their homogenates]. After 1 h reaction, 10 μl of the reaction mixture was withdrawn and NADP in it was enzymatically determined with isocitrate dehydrogenase (5). Activity was expressed as μmol/g-cell/h.

Analysis of reaction products. The NADP-producing reaction was carried out by using purified Ppnk and immobilized cells under the conditions described above in the presence of 50 mg/ml metaphosphate or 50 mM ATP. After 24 h reaction, each of the reaction mixtures was diluted by 50 fold with 50 mM Tris-acetate (pH 7.5), and the diluent (30 μl) was applied to a TSK-GEL ODS-80TS column (0.46 x 15.0 cm) (Tosoh, Tokyo, Japan) and the adsorbed nucleotides were eluted through an increasing gradient of methanol from 0 to 10 % in 50 mM Tris-acetate (pH 7.5) (0 – 40 min : 0 – 10 %, 40 – 50 min : 10 %) at the flow rate of 0.70 ml/min. Elution positions of nucleotides were determined by measuring absorbance at 260 nm.

Analysis of metaphosphate. Metaphosphate (10 %, 60 ml, pH 7.0) was dialyzed at 25 ℃ for 24 h with Seamless Cellulose Tube (Exclusion size: 12,000 - 14,000 Da) (Viskase Sales Corp, Chicago, IL) against 3,000 ml of water. The outer solution (2,900 ml) was passed through Dowex 1x2 (Cl^-, 200-400 mesh) column (3.0 x 6.0 cm) (Muromachi Kagaku Kogyo, Tokyo, Japan), and adsorbed phosphate polymers were then eluted with a liner gradient of LiCl (600 ml, 0-1.0 M, pH 2.0) in 6.0 ml fractions every 6.0 min. A portion (0.10 ml) of each fraction was used for the assaying of the poly(P)-dependent NAD kinase activity of Ppnk as described above, and the amount of NADP produced for 1 min was defined as phosphoryl donor activity. Acid-labile phosphates in each fraction were estimated by determining inorganic orthophosphates released from metaphosphate after boiling the eluates in 1 N HCl for 7 min (11).

RESULTS

NADP-producing activity of Ppnk. *M. tuberculosis* Ppnk was overexpressed in *E. coli* SK27 cells having an ATP-NAD kinase. The cell extract of SK27 showed 31 units/mg of poly(P)-dependent NAD kinase activity, which was approximately 400-fold higher than that of *B. ammoniagenes* (0.075 units/mg), which was previously used to produce NADP from NAD and poly(P) (metaphosphate) (4). Ppnk (90 units/mg) purified from the cell extract of SK27 (7) was used for the production of NADP. When 14.4 units of the purified Ppnk was used, 30 mM (22 g/l) NADP was produced from 50 mM NAD and 100 mg/ml metaphosphate (Fig. 1A). However, conversion of NAD to NADP remained at less than 60 % irrespective of the metaphosphate concentration (Fig. 1A). The low conversion was considered to be caused by the inhibition of the poly(P)-dependent NAD kinase activity of Ppnk by NADP produced. In fact, the poly(P)-dependent NAD kinase activity of Ppnk was significantly inhibited by NADP, but not by ADP, the inhibition being almost complete with 30 mM NADP (Fig. 1B).

NADP-producing activity of immobilized cells. NADP production with the use of purified Ppnk has some disadvantages: (i) The purification of Ppnk is tedious and intricate, (ii) the stability of Ppnk is not so high, and (iii) re-use of the enzyme is impossible (unless it is insolubilized). The Ppnk was immobilized on an ion-exchange resin, and then used for the continuous production of NADP from NAD and metaphosphate. Although the detailed conditions for NADP production are not presented here due to a patent application, the immobilized enzyme (Ppnk) system is now being operated on an industrial scale.

However, the use of immobilized cells is supposedly more convenient than that of immobilized enzymes, since purification of the enzyme is unnecessary and a substrate solution of high ionic strength can be applied. Then SK27 cells overexpressing Ppnk were immobilized in a polyacrylamide gel lattice and treated with acetone to increase the permeability of substrates [NAD and poly(P)n] and/or products [NADP and poly(P)n-1]. A summary of the NADP-producing activities of various SK27 cell preparations is shown in Table 1. The NADP-producing activity of the cell homogenate was 1,530 μmol/g-cell/h (p). On the other hand, that of the immobilized cell homogenate was 925 μmol/g-cell/h (q), which indicates that about 60 % [(q/p)x100] of the NADP-producing activity initially present in the intact cells was entrapped in the polyacrylamide gel. Furthermore, the activity (843 μmol/g-

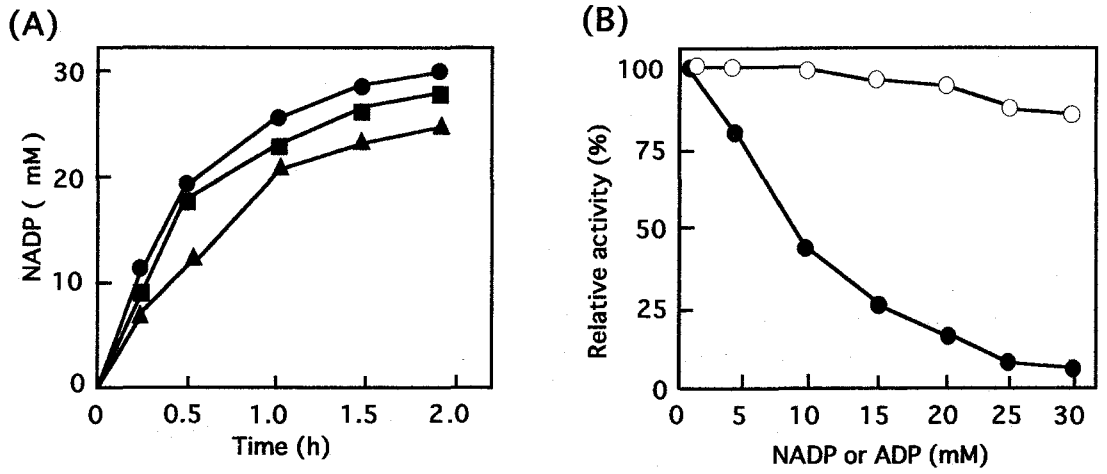

FIG. 1 NADP-producing activity of purified Ppnk. (A) Effects of the metaphosphate concentrations on the NADP-producing activity of purified Ppnk. NADP-producing activity was assayed as described in MATERIALS AND METHODS in the presence of 50 (■), 100 (●), or 150 (▲) mg/ml metaphosphate. (B) Effects of the NADP and ADP concentrations on the NADP-producing activity of purified Ppnk. The NADP-producing reaction was performed as described in MATERIALS AND METHODS in the presence of various amounts of NADP (●) or ADP (○).

cell/h) of the homogenate of acetone-treated immobilized cells was higher than that (672 μmol/g-cell/h) in the case of acetone-treated immobilized cells. This indicates that a polyacrylamide gel lattice is a barrier for the transport of substrates and/or products.

TABLE 1 NADP-producing activities of various cell preparations

Preparation	NADP-producing activity[a] (μmol/g-cell/h)
Intact cells	93.5
Homogenate of intact cells (*p*)	1,530
Acetone-treated cells	642
Homogenate of acetone-treated cells	1,420
Immobilized cells	146
Homogenate of immobilized cells (*q*)	925
Acetone-treated immobilized cells	672
Homogenate of acetone-treated immobilized cells	843

[a]NADP-producing activity was determined as described in MATERIALS AND METHODS with metaphosphate as a phosphoryl donor.

Properties of Ppnk in immobilized cells. The properties of Ppnk in acetone-treated immobilized cells were examined and compared with those in the case of acetone-treated cells. (i) *Temperature effect.* Heat treatment at 60 ℃ for 10 min was required to abolish 50 % of the NADP-producing activity of Ppnk in acetone-treated immobilized cells, while treatment at 50 ℃ was enough to obtain 50 % inactivation of the Ppnk activity in acetone-treated cells (Fig. 2A), showing that the heat stability of Ppnk was enhanced on immobilization in a polyacrylamide gel lattice. The optimum temperature for the NADP-producing activity of Ppnk shifted from 50 ℃ to 55 ℃ on immobilization (Fig. 2B). (ii) *pH effect.* The optimum pH for the NADP-producing activity of Ppnk in acetone-treated immobilized cells was 7.0, i.e. slightly higher than that (pH 6.5) of Ppnk in acetone-treated cells (Fig. 2C). (iii) *Operational stability.* Acetone-treated immobilized cells were repeatedly used for assaying of the NADP-producing reaction, and the operational stability of the NADP-producing activity of Ppnk was compared with that in the case of Ppnk in acetone-treated cells similarly used (Fig. 2D). The activity of Ppnk in acetone-treated cells was almost completely lost after repeated use 5 times, while that of Ppnk in acetone-treated immobilized cells was maintained at a nearly constant level.

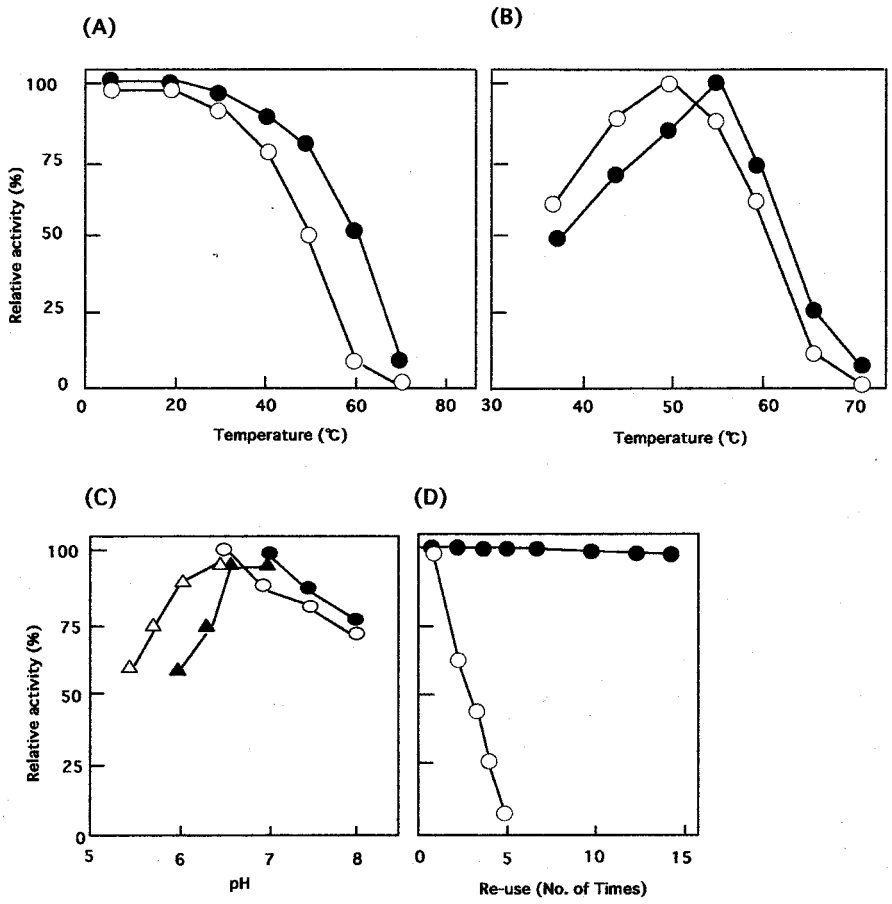

FIG. 2 Properties of Ppnk in acetone-treated immobilized cells. The properties of Ppnk in acetone-treated immobilized cells were examined by determining NADP-producing activity and compared with those of acetone-treated cells. The maximum NADP-producing activity of Ppnk in each cell type was taken as 100 %. (A) Heat stability. Acetone-treated immobilized cells (●) or acetone-treated cells (○) were incubated in 0.5 ml of 5.0 mM Tris-HCl (pH 7.0) at various temperatures for 10 min, and then the residual NADP-producing activity was determined as described in MATERIALS AND METHODS. (B) Optimum temperature. Acetone-treated immobilized cells (●) or acetone-treated cells (○) were incubated as described in MATERIALS AND METHODS at various temperatures. (C) Optimum pH. Acetone-treated immobilized cells (▲, ●) or acetone-treated cells (△, ○) were incubated as described in MATERIALS AND METHODS in 100 mM sodium acetate (▲, △) or Tris-HCl (●, ○). (D) Operational stability. Acetone-treated immobilized cells (●) or acetone-treated cells (○) were repeatedly used for the assaying of NADP-producing activity as described in MATERIALS AND METHODS. Each time after 1 h, the cells were washed with 5.0 mM Tris-HCl (pH 7.0) and then used again for the reaction in a freshly prepared reaction mixture.

The half-life of the Ppnk activity in acetone-treated immobilized cells was calculated to be more than 75 days, although the results of long-term experiments are not shown here.

Production of NADP by immobilized cells. The conditions for NADP production by acetone-treated immobilized cells were investigated (Fig. 3 A, B, C, D). (i) *Metaphosphate concentration.* The NADP-producing activity of Ppnk in acetone-treated immobilized cells initially increased with an increase in the metaphosphate concentration, reached plateau at 100 mg/ml, and then decreased gradually (Fig. 3A). A similar activity-substrate concentration relationship was observed in the case of ATP, the NADP-producing activity being maximum with 150 mM ATP ([ATP]:[Mg^{2+}] = 3:2] (Fig. 3B). (ii) *NAD concentration.* The NADP-producing activity of Ppnk in acetone-treated immobilized cells increased in proportion to the amount of NAD (Fig. 3C), although that at more than 50 mM NAD was not determined. (iii) *Metal ion concentrations.* Among the effective metal ions (Mg^{2+}, Mn^{2+}, and Ca^{2+}) for the poly(P)-dependent NAD kinase activity of Ppnk (7), Mg^{2+} was the most suitable for the NADP-producing activity of Ppnk in acetone-treated immobilized cells, the maximum activity being attained with 100 mM Mg^{2+} in the presence of 100 mg/ml metaphosphate and 50 mM NAD (Fig. 3D). Mn^{2+} and Ca^{2+} formed precipitates at more than 5.0 mM.

When acetone-treated immobilized cells were incubated in the optimum reaction mixture [50 mM NAD, 100 mg/ml metaphosphate, (or 150 mM ATP (Fig. 4B)), 100 mM MgCl2, and 100 mM Tris-HCl (pH7.0)], the maximum amount of NADP, 16 mM (12 g/l), was produced (Fig. 4A), while immobilized SK45 cells produced no or only a small amount of NADP under the same conditions (Fig. 4A, B). The low conversion (about 30 %) of NAD to NADP was thought to be due to the inhibitory effect of NADP produced (Fig. 1B) and/or restriction of the diffusion of products or substrates by the polyacrylamide gel lattice, as discussed above, since degradation of NAD and NADP by acetone-treated immobilized cells was negligible (data not shown). The removal of NADP from the reaction system should result in improvement of the transformation efficiency. The amount of NADP produced by immobilized SK27 cells was significantly higher than that of NADP (2.0 mM, 1.5 g/l) obtained with immobilized *B. ammoniagenes* cells (4). Furthermore, the maximum productivity of NADP (16 mM, 12 g/l) (Fig. 4A) in the presence of metaphosphate (100 mg/ml) was comparable with that in the presence of ATP (150 mM) (Fig. 4B). This emphasizes that the NADP production systems developed here with the purified Ppnk and/or immobilized recombinant *E. coli* cells are highly efficient and economical compared with that with ATP-NAD kinase (2).

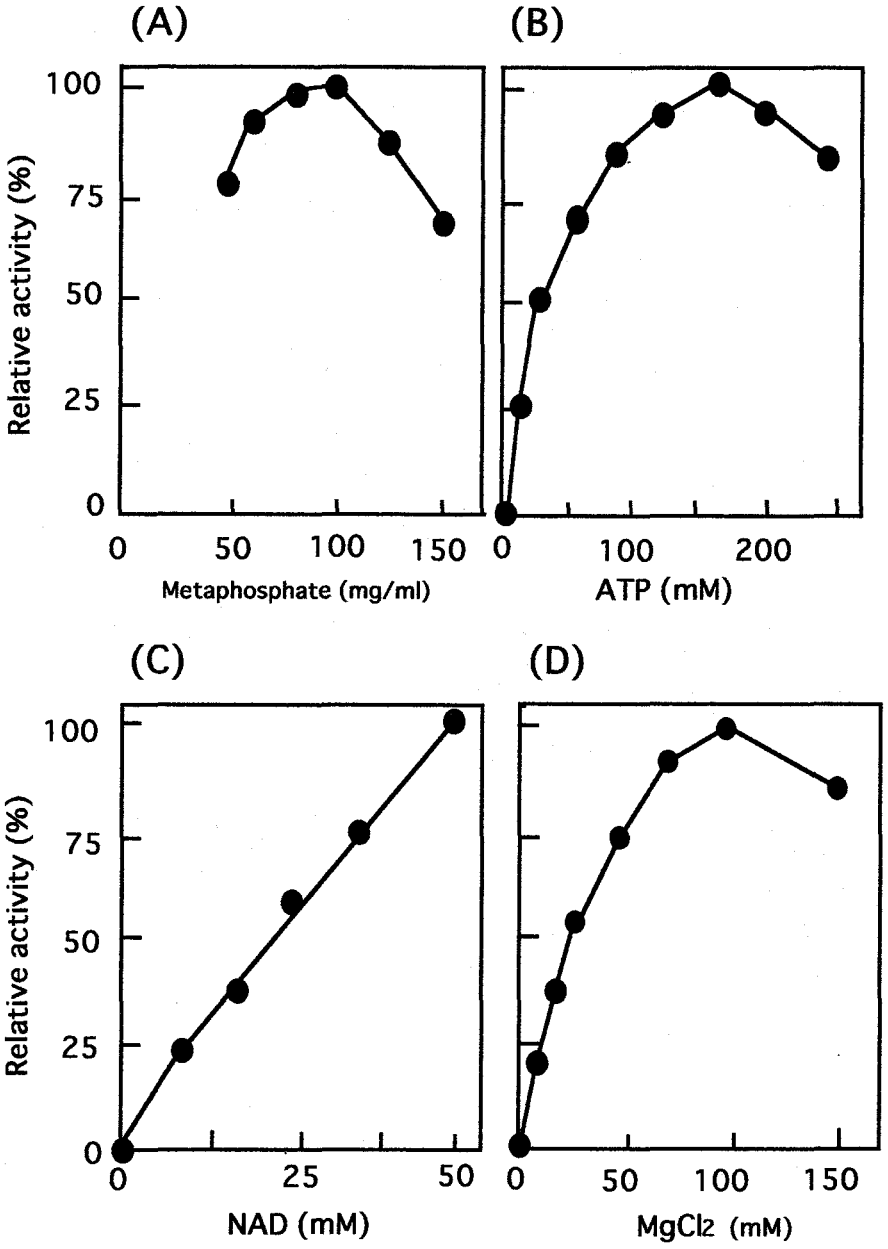

FIG. 3 Properties of Ppnk in acetone-treated immobilized cells. The NADP-producing activity of Ppnk in acetone-treated immobilized cells was determined as described in MATERIALS AND METHODS, except that the amounts of (A) metaphosphate, (B) ATP, (C) NAD, and (D) Mg^{2+} were varied. In (A), precipitates formed at less than 40 mg/ml metaphosphate. In (B), ATP was used instead of poly(P). The maximum NADP-producing activity of Ppnk was taken as 100 %.

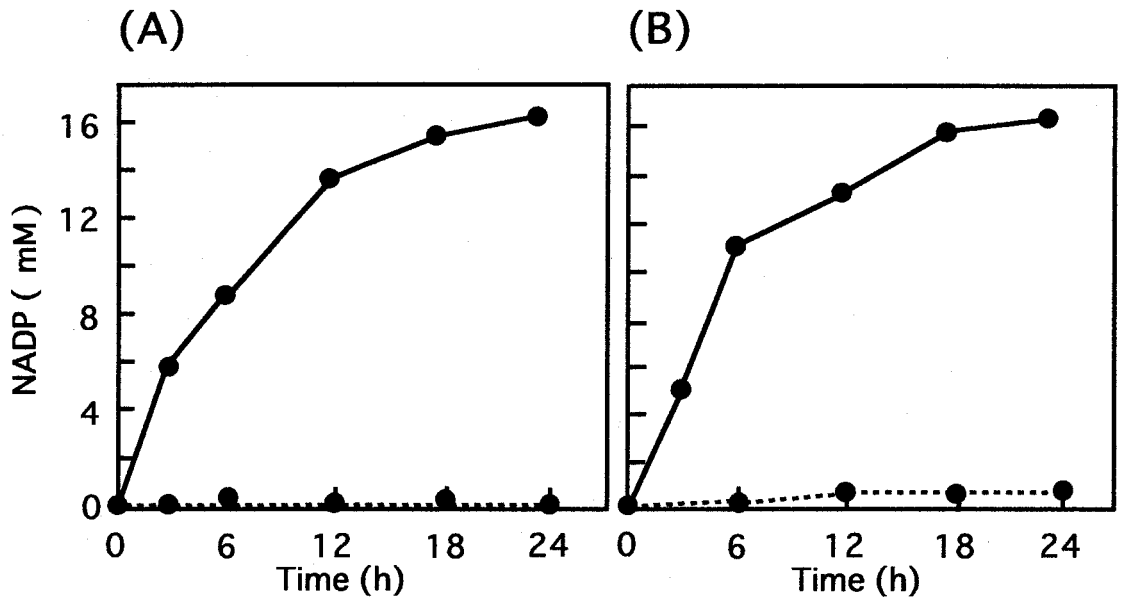

FIG. 4 NADP production by acetone-treated immobilized SK27 (solid lines) and SK45 (dotted lines) cells. The NADP-producing reaction was performed in the optimum reaction mixture (4.0 ml) consisting of 50 mM NAD, 100 mM $MgCl_2$, 100 mM Tris-HCl (pH7.0), immobilized cells (0.10 g), and 100 mg/ml metaphosphate (A) or 150 mM ATP (B). At the indicated times, 10 μl of the reaction mixture was withdrawn and the amount of NADP in the mixture was determined as described in MATERIALS AND METHODS.

Analysis of reaction products. The NADP-producing reactions were carried out using purified Ppnk and immobilized cells in the presence of ATP or metaphosphate, and the changes in components were examined (Fig. 5). When ATP and purified Ppnk were used, the mixture after reaction contained NADP and ADP in addition to the unreacted ATP and NAD (Fig. 5B). When ATP and immobilized cells were used, that contained NADP, ADP, and AMP in addition to the unreacted ATP and NAD (Fig. 5C). Formation of adenosine was also detected by thin layer chromatography (data not shown). On the other hand, when metaphosphate was used instead of ATP, the mixture after reaction consisted of only NADP and unreacted NAD in both cases of purified Ppnk (Fig. 5E) and immobilized cells (Fig. 5F).

Phosphoryl donor substrates in metaphosphate. Metaphosphate is a mixture of ring and/or linear phosphate polymers with different polymerization degrees. To know the intrinsic phosphoryl donor substrates for the poly(P)-dependent NAD kinase activity of Ppnk, phosphate polymers in metaphosphate were separated by ion-exchange column chromatography as described in MATERIALS AND METHODS (Fig. 6). When metaphosphate was dialyzed against water, approximately 84 % of phosphoryl donor activity was recovered in outer solution, indicating that the greater part of substrates for the enzyme are phosphate polymers with a molecular mass less than 12,000 – 14,000 Da. When fractionated the outer solution on Dowex column, phosphoryl donor activities were detected in all the fractions eluted at more than 0.20 M LiCl (fraction nos. 25-80) (Fig. 6). Although the results obtained gave no information regarding the structure and/or polymerization degrees, they indicate that the metaphosphate contains at least four kinds of phosphoryl donor substrates for the poly(P)-dependent NAD kinase activity of Ppnk.

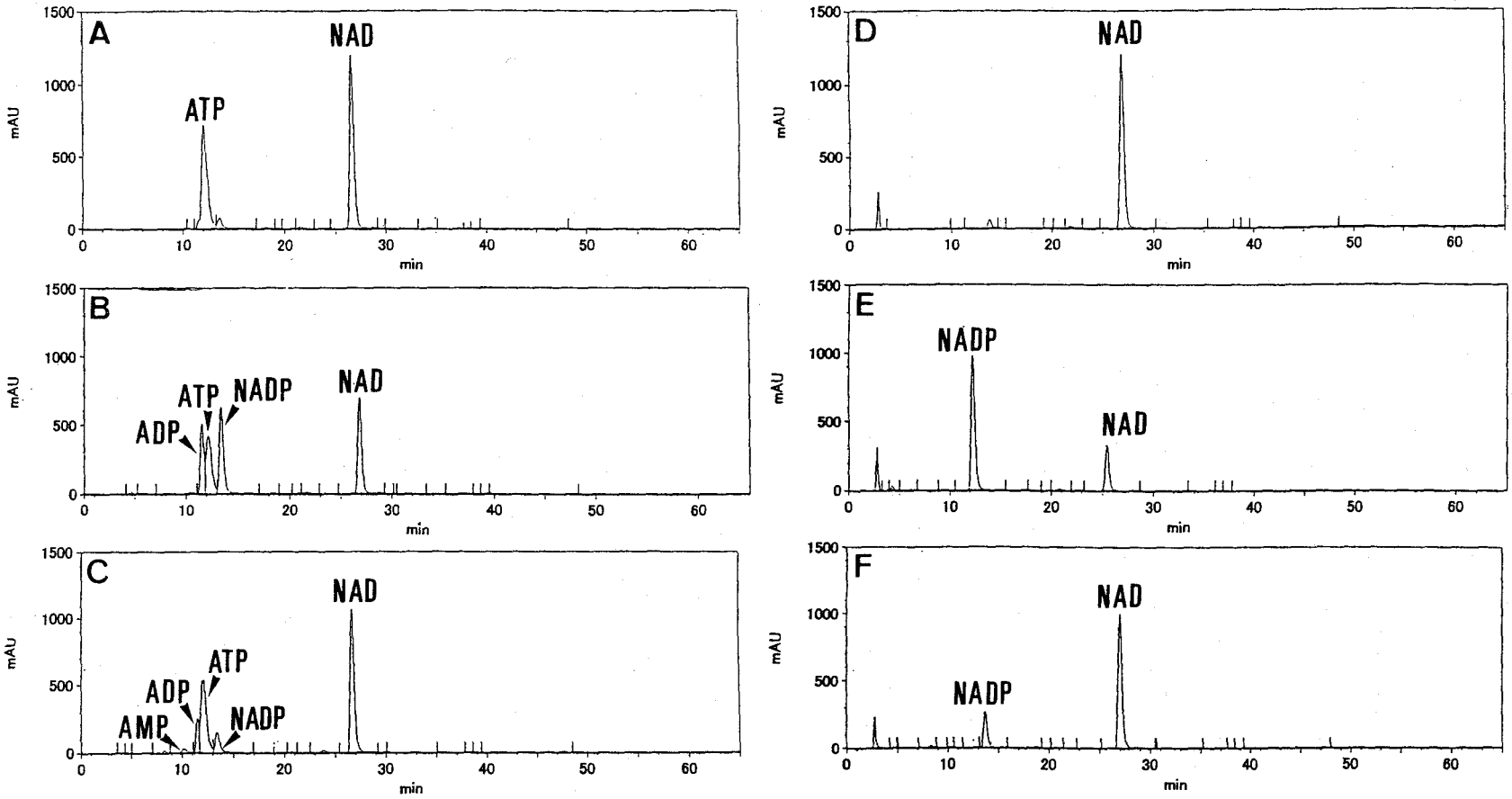

FIG. 5. Analysis of reaction products. The NADP-producing reactions were carried out using purified Ppnk (B and E) and immobilized cells (C and F) in the presence of ATP (left panels) or metaphosphate (right panels), and changes in components before (A and D) and after reactions were analyzed as described in MATERIALS AND METHODS.

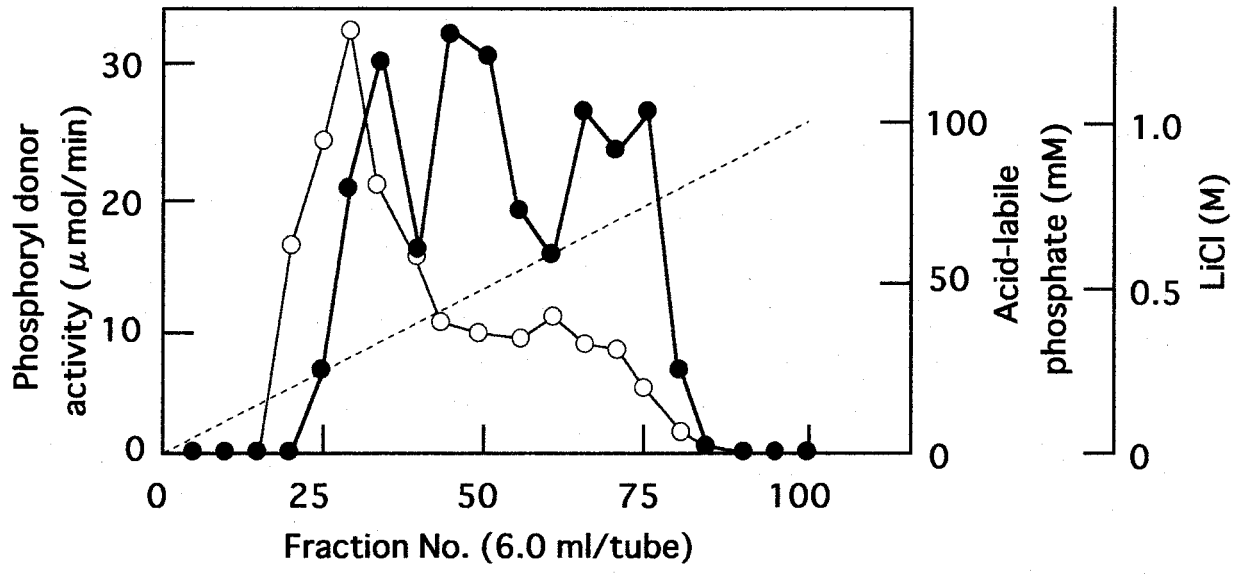

FIG. 6 Elution pattern of phosphoryl donors in metaphosphate on Dowex 1x2 column. Phosphate polymers in dialysate (outer solution) was passed through the Dowex 1x2 column and adsorbed phosphate polymers were eluted with a liner gradient of LiCl from 0 to 1.0 M (pH 2.0) as described in MATERIALS AND METHODS. ●, Phosphoryl donor activity; ○, acid-labile phosphate; ----, concentration of LiCl.

DISCUSSION

Up to now, the industrial application of microbial enzymes has been limited to the catalysis of degradation and simple transformation reactions, and extensive application of enzymes to synthetic reactions requiring an energy (ATP) supply has not been performed on an industrial scale. The barrier preventing the development of an economically feasible ATP-requiring process may be the lack of an adequate (re)generation and/or recycling system for ATP, and the construction of a (re)generation system for ATP is therefore indispensable not only for the economic utilization of enzymes, but also for process economy and reaction efficiency. For this purpose, various routes to ATP (re)generation involving chemical synthesis, whole cells, organellar or sub-cellular systems, and cell free enzymes have been evaluated (12). However, despite the great strides made in the last decade, the technological and economical feasibility of these routes as ATP regeneration systems remains uncertain, except for the use of subcellular systems (glycolysis) (13, 14).

A production system involving poly(P) as a phosphoryl donor, as developed here, is a promising alternative to the use of ATP-requiring enzymes for the production of useful compounds, for the following reasons : [1] – [4]. [1] The production system is highly economical, since poly(P) is obtained at a nominal price, and it contains sufficient substrates for the poly(P)-dependent NAD kinase activity of Ppnk (Fig. 6). [2] Isolation of products (NADP) is easy, since the system contains no ATP degradation products (Fig. 5). [3] Various kinds of enzymes that utilize poly(P) as an energy source are present in microbes (6, 15, 16), and some of them are readily applicable to production of useful chemicals. For example, nucleoside-5'-monophosphate was produced from nucleoside and pyrophosphate with *Morganella morganii* (15, 16), and glucose-6-phosphate was from glucose and metaphosphate with an immobilized *Achromobacter butyri* cell column (11). [4] Given our present knowledge and the state of (bio)technology, ATP-dependent kinases in the present organisms can be converted to poly(P)-dependent kinases through gene and protein engineerings. Our recent research indicates that, although at an extremely low level, the ATP-NAD kinase in *E. coli* also possesses poly(P)-dependent NAD kinase activity, and that the nucleotide sequences of ATP-NAD kinases of *E. coli* and *Saccharomyces cerevisiae* are closely similar to those of poly(P)/ATP-NAD kinases from *Micrococcus flavus* and *M. tuberculosis* H37Rv (Chapter III). Taking into consideration that a biochemical energy carrier may originate from poly(P) (17), the nucleotide sequence similarity suggests that the ATP-dependent kinases have developed

from poly(P)-dependent ones, possibly through the accumulation of point mutations, thus suggesting that a creation of an NAD kinase that accepts poly(P) instead of ATP would be possible. The same strategy may be applied to the conversion of other ATP-dependent kinases to poly(P)-dependent ones.

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CONCLUSION

1. A novel enzyme "poly(P)/ATP-NAD kinase" was purified from *Micrococcus flavus* that utilizes both poly(P) and ATP as phosphoryl donors. Physiological role of poly(P)-dependent NAD kinase activity of the enzyme was evidenced through an isolation of intrinsic poly(P) substrates from *M. flavus*.

2. NAD kinase was purified from *Escherichia coli* that uses ATP, but not poly(P), and designated as "ATP-NAD kinase". Based on the results of 1. and 2., it was revealed that at least two kinds of NAD kinases "poly(P)/ATP-NAD kinase" and "ATP-NAD kinase" exist in biological systems.

3. Poly(P)/ATP-NAD kinase genes were cloned from *M. flavus* (*mfnk*) and *Mycobacterium tuberculosis* H37Rv (*ppnk*), and ATP-NAD kinase genes from *E. coli* (*yfjB*) and *Saccharomyces cerevisiae* (*UTR1*). Despite of the difference of phosphoryl donor specificities, the primary structures of these NAD kinases were highly conserved. Ppnk was crystallized and its crystal parameters were determined.

4. Bacterial Mfnk, and Ppnk, and yeast Utr1p showed NADH kinase activities, while *E. coli* YfjB did not and was inhibited allosterically by NADH and NADPH, thus suggesting that different regulation mechanisms for NAD(P)H synthesis are operating in microorganisms.

5. Mass production system of NADP with Ppnk was established and the method industrialized.

PUBLICATION LIST

1. Kawai, S., Mori, S., Mukai, T., and Murata, K. (2001) Bacterial poly(P)/ATP-NAD kinase is a sole enzyme prerequisite for NADP and NADPH biosynthesis.
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ACKNOWLEDGEMENTS

The author wishes to express his most grateful acknowledgement to Dr. Kousaku Murata, Professor of Graduate School of Agriculture, Kyoto University, for his kind guidance and warm encouragement during his study.

The author is deeply indebted to Dr. Wataru Hashimoto and Dr. Bunzo Mikami, Associate Professors of Graduate School of Agriculture, Kyoto University, for their helpful advice and useful suggestions.

The author is also deeply indebted to Dr. Hidehiko Kumagai and Dr. Kenzi Yamamoto, Professors of Graduate School of Biostudies, Kyoto University, for their warm encouragements during his research.

The author would like to express deep gratitude to Dr. Takeshi Yamada, School of Dentistry, Nagasaki University, for his gift of genomic DNA of *Mycobacterium tuberculosis* H37Rv, and to Mr. Hirokazu Matsukawa and Dr. Yuhsi Matuo, Oriental Yeast Co., Ltd., to their useful discussion, invaluable advice, and assistance in carrying out the study of NADP production.

Thanks are given to the members of the Department of Basic and Applied Molecular Biotechnology, Graduate School of Agriculture, Kyoto University, for their helpful collaborations and encouragement.